Université des Antilles et de la Guyane

Faculté de Sciences Exactes et Naturelles

École doctorale pluridisciplinaire

Thèse pour le doctorat en Sciences de la Vie

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Acquisition de connaissances sur la génétique de l'espèce Dioscorea alata L. pour la production de variétés polyploïdes

Sous la direction de Jacques DAVID et Amadou BA

Soutenue le 29 Juin 2012 à l'Université des Antilles et de la Guyane

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A mes parents Alexandrine CECILIA NEMORIN et Joël NEMORIN qui ont été mes épaules, au fruit de mes entrailles mon fils Alan KANDASSAMY—NEMORIN qui a été mon cœur, à mon compagnon Alain KANDASSAMY qui été ma côte, à mes frères et sœurs Michel, Fanny et Maxime qui ont été mes bras droits et à mon souffle, l'Invisible omniprésent…

« On cultivait la fleur d'igname car on disait qu'elle réjouissait le cœur des bons esprits. Mais rares étaient ceux qui s'y essayaient. La plante en effet était délicate, poussait difficilement, et de plus, ses feuilles, même cuisinées, ne se mangeaient pas. »

Isabelle REVOL, 2001, p.5. Fleur d'igname, Nouméa, Éditions Catherine Ledru, 24 p.

Demeurez toujours attachés à vos racines…

Remerciements

Ce chapitre a débuté un beau matin en 2007 où Mr Daugrois m'a suggéré de faire une thèse alors que j'avais déjà renoncé à cette idée. Je l'en remercie et je remercie également :

Gemma ARNAU qui m'a proposé ce magnifique sujet et qui m'a encadré, pas dans le sens dirigé mais plutôt entouré pendant ces trois années et même avant pendant mes deux années de volontariat. Elle a vraiment été mon carburant c'est elle qui m'a poussé, boosté pendant ces 5 années, qui a eu confiance en moi et qui a su peu à peu me donner confiance en moi.

La Région Guadeloupe qui a accepté de financer mon projet de thèse et de façon globale mon projet professionnel car elle m'a également soutenue financièrement lors de mes études.

Le CIRAD qui a été mon laboratoire d'accueil et plus précisément Mr Hainzelin directeur de la recherche et de la stratégie au CIRAD pour l'appui financier, Mr Glaszmann, Mr Domaingue et Mme Duval pour leur accueil au sein de leur département, unité et équipe respectivement.

L'Université des Antilles et de la Guyane Française et son président MR Saffache pour l'intégration au sein de son université et Mme Abaul pour l'intégration au sein de son Ecole doctorale.

Jacques DAVID pour sa direction, sa disponibilité malgré un emploi du temps très chargé, pour sa présence aux réunions de travail malgré la distance et les contraintes de la visioconférence, pour ces compétences pluridisciplinaires , pour son expertise scientifique et pour son soutien et ses corrections lors du sprint final malgré ses autres obligations.

Mr BA pour sa codirection et pour tous ses conseils pour un bon déroulement de ma thèse.

Les membres de mon comité de thèse Mr Bakry, Mme D'hont, Mr Perrier, Mr Jenczewski pour l'appui scientifique dans leurs domaines de compétences respectifs.

Les membres du jury, Mme Chèvre, Mr Dansi, Mr Rousteau et Mme Scarcelli qui ont accepté d'évaluer mon travail en 2012.

Je remercie par ailleurs l'Equipe Igname, permanents et passagers :

Gemma ARNAU de nouveau pour sa passion, son enthousiasme communicatif et ses petites répliques cultes : « ça vaut de l'or », « Champagne », « C'est super »

Malédon Erick pour sa collaboration et pour son aide.

Nudol Elie pour s'être bien occupé de mon matériel végétal et de mes parcelles et pour son soutien moral.

Ceux qui étaient de passage durant mon quinquennat mais qui ont tout de même laissé leur trace :

Frédéric Lambert pour son humour inouï et sa bonne humeur en toutes circonstances et pour sa motivation dans son travail et pour les autres. Toujours positif !

Adrien Denis pour les expériences qu'il a mené dans le cadre de ma thèse avec rapidité et efficacité

Les chercheurs indiens : Mr Abraham grand spécialiste de cette espèce pour les connaissances transmises et Mme Sheela pour son partenariat, sa bienveillance et sa sagesse.

Les stagiaires Mattew Valley a qui j'ai fait faire des kilomètres, Audrey Paulo et Jessica Dalon que j'ai encadré, Fiorella Fanton pour son très fameux gâteau à l'igname et aussi les autres stagiaires canadiens que j'ai côtoyé.

Tous les collègues de Roujol : Les chercheurs, Mr Daugrois qui répondait à toutes mes questions d'ordre administratif et scientifique, Mr Hoareau pour les longues discussions et l'animation scientifique, les thésardes Carine Edon qui a été la grande sœur que je n'ai pas eue, Sarah Debibakas qui est devenue une amie, sans oublier Solène Rocher pour ses encouragements, les stagiaires Séverine Miatti-Tormin et Elvire Couchy pour leur bonne humeur.

Les techniciens, en particulier Georges Gelabale pour sa galanterie et Nadia Adjanoh-Lubin pour m'avoir communiqué l'envie de démarrer un autre projet que celui de la thèse.

Les autres collègues du CIRAD Roujol, de l'administration Annick Anette, Sylvie Perrot qui m'a aidé à faire mes nombreux pots de départ et oui j'en ai fait beaucoup en 6 ans, du laboratoire Rosiane Boise-Noc, Marie-Claire Gravillon, Jocelyne Sapotille, Chantal Guiougou et Patricia Navis et du terrain Jean-Marie Coupan, Sylvere Carmel et les autres ; malheureusement je ne peux pas tous vous citer mais je vous remercie tous pour la bonne ambiance générale sur la Station.

Les collègues du CIRAD EMVT pour leur accueil lors de nos travaux de cytométrie en flux et particulièrement Nathalie Vachiéry, Rosalie Aprelon et Loïc Emboulé.

Les collègues du CIRAD Neuf-château, plus particulièrement ceux du laboratoire Guyot, également pour leur accueil lors des longues journées d'extractions et de PCR, je pense à Pierre-Yves Techeney, Didier Beguié-M-Béguié, Isabelle Acina, Sandrine Giougoux et Marie Humbert.

Philippe Godon, Directeur régional du CIRAD, pour avoir facilité les démarches administratives.

Mes interlocuteurs des plateformes de génotypage de Pointe-à-pitre Cédric Broquere, de l'INRA de Montpellier Sylvain Santoni, de l'INRA de Clermont-Ferrand Charles Poncet.

Les responsables du département de biologie Mr Guerlotté et Mme Gustave pour m'avoir fait confiance dans l'expérience d'enseignement ainsi que les professeurs avec lesquels j'ai collaboré Mme Lemoine, Mme Dulorme, Mr Rousteau et Mr Imbert.

Ma copine-sœur Malyka Kancel pour son soutien.

Je finirais par des remerciements à tous les membres de ma famille et de ma belle-famille qui m'ont soutenu d'une manière ou d'une autre familles CECILIA en particulier Gladys Chapiteau, NEMORIN et KANDASSAMY.

Résumé

Dioscorea alata est un complexe polyploïde avec des niveaux de ploïdie allant de diploïde ($2n = 2x = 40$) à tétraploïde ($2n = 4x = 80$). Chez cette espèce l'augmentation de la ploïdie est corrélée à une augmentation de la vigueur et à des rendements plus élevés et plus stables. L'objectif de cette thèse est d'acquérir des connaissances sur les phénomènes de polyploïdisation des *D .alata* en vue d'optimiser les stratégies de création d'hybrides polyploïdes.

Différentes approches ont été utilisées afin de déterminer le type de ségrégation des variétés tétraploïdes : des analyses d'hérédité de marqueurs microsatellites à l'aide de la méthode Bayésienne, l'observation de phénomènes de double réduction et l'étude des méioses des cellules mères de pollen. Les résultats obtenus ont permis de démontrer que les variétés tétraploïdes ont un type de ségrégation polysomique et sont autotétraploïdes.

Nous avons ensuite déterminé les mécanismes les plus probables à l'origine des polyploïdes naturels via l'étude de la transmission de l'hétérozygotie parentale à l'aide de microsatellites et par l'étude des incompatibilités au niveau de l'albumen lors des différents croisements intracytotypes et intercytotypes en utilisant la cytométrie en flux.

Les résultats obtenus montrent que les polyploïdes de *D. alata* seraient apparus via la formation de gamètes non réduits de clones diploïdes. Le pool triploïde se serait édifié et diversifié uniquement par formation de $2n$ gamètes chez des femelles diploïdes, du fait de la non viabilité des croisements avec formation de diplogamètes chez les mâles et des croisements intercytotypes. Le pool tétraploïde serait apparu par union de deux gamètes non réduits de clones diploïdes (polyploidisation sexuelle bilatérale). Par la suite ce pool aurait été diversifié via des croisements intercytotypes avec formation de $2n$ gamètes par voie femelle et par voie mâle, ainsi que par croisements intracytotypes au sein du pool $4x$.

Ce travail a permis d'acquérir des connaissances importantes sur l'origine et le type de ségrégation des polyploïdes qui permettront d'optimiser les stratégies d'amélioration chez cette espèce.

Mots clés : *Dioscorea alata*, Polyploïdie, ségrégation polysomique, $2n$ gamètes, origine des triploïdes.

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INTRODUCTION

L'igname

Les ignames sont des monocotylédones appartenant à la famille des Dioscoréacées et au genre *Dioscorea*. Ce sont des lianes herbacées volubiles à tubercules, produisant dans certains cas des bulbilles. Ces espèces bien que monocotylédones sont riches en traits de dicotylédones tel que la présence de pétioles, la nervation du limbe et l'organisation des faisceaux libéro-ligneux en un seul cercle dans les jeunes tiges (Degras 1993). Par ailleurs, la formation du pollen par division simultanée témoigne de cette proximité avec les Dicotylédones (Essad 1984). La formation du sac embryonnaire serait de type Onagracée pour *D. villosa* d'après Smith 1916, de type Polygonacée pour *D. oppositifolia* d'après Rao 1953. Des travaux plus récents ont démontré que le sac embryonnaire est de type *Polygonum* (figure 1) chez l'espèce *D. nipponica* (Torshilova et al. 2003).

Le genre Dioscorea créé en 1753 par Linné représente à lui seul plus des 90% des espèces qui composent cette famille. Il regroupe plus de 600 espèces (Ayensu et Coursey l972). Il existe uniquement 10 espèces cultivées dont les majeures sont : *Dioscorea rotundata et Dioscorea cayenensis* originaires d'Afrique de l'Ouest, *Dioscorea alata* (figure 2, 3, 4) originaire d'Asie du Sud –Est et du Pacifique Sud et *Dioscorea trifida* originaire d'Amérique du Sud. Une représentation sur une carte des centres d'origine et de diversification des différentes espèces est donnée en figure 5. Les trois premières appartiennent à la section Enantiophyllum et se caractérisent par des lianes s'enroulant dans le sens antihoraire et des feuilles entières. Tandis que l'espèce *D. trifida* appartient à la section Macrogynodium et se caractérise par des feuilles lobées et des lianes s'enroulant dans le sens horaire. Les espèces cultivées mineures sont *D. esculenta*, cultivée en Asie du Sud-Est et en Mélanésie, *Dioscorea opposita-japonica*, cultivée en Chine et au Japon, *D. transversa* en Australie et en Mélanésie, *D.nummularia* en Indonésie et dans le Pacifique, *D. bulbifera* et *D. pentaphylla* (Annexe 1). Il existe aussi plusieurs espèces sauvages qui constituent une source alimentaire importante pour certaines populations, telles que les espèces endémiques de Madagascar *D. soso*, *D. nako*, *D. bemandry*, *D .alatipes*, *D .hombuka* et l'espèce *D. hamiltonii* en Inde.

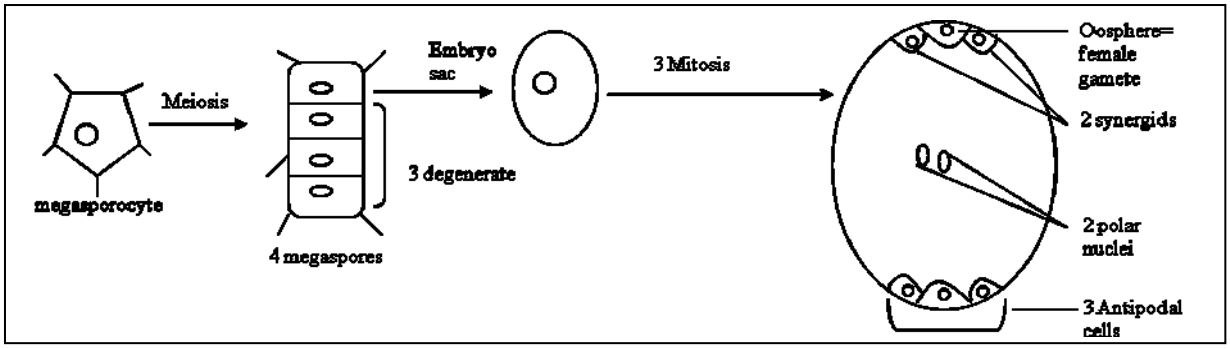

Figure 1 Mégasporogénèse et mégagamétogénèse aboutissant à la formation d'un sac embryonnaire type Polygonum

Figure 2 Appareil végétatif des trois cytotypes de *D. alata*. De gauche à droite cytotype diploïde, triploïde et tétraploïde

Figure 3 Feuilles des trois cytotypes de *D. alata*. De gauche à droite cytotype diploïde, triploïde et tétraploïde

Figure 4 Tubercules de *D. alata*

Figure 5 Centres d'origine et de diversification des quatre espèces cultivées majeures

Les ignames ont une grande valeur nutritive en raison de leur richesse en glucides, en fibres alimentaires, en minéraux et en vitamines. Ces tubercules sont composés en grande partie d'amidon (68% de la matière sèche en moyenne), de protéines (6%) et de sucres (6%). Ils sont très riches en potassium (816 mg pour 100 g), en phosphore, en calcium et en oligoéléments manganèse, zinc, cuivre. Ils sont très riches en vitamine E (0,39 mg pour 100 g) et en vitamines K (2,6 mg pour 100g) et riches en vitamines C, B6 et B1. Ces tubercules contiennent une molécule la dioscorine à qui on attribue des effets antioxydants et hypotenseur.

Les ignames sont d'une grande importance économique et alimentaire dans les régions tropicales puisqu'elles constituent la base de l'alimentation de plus de 300 millions de personnes à travers le monde (FAOSTAT, 2008). L'Afrique de l'Ouest et l'Afrique Centrale contribuent à elles seules, à plus de 90% de la production mondiale (figure 6) estimée à 56,4 millions de tonnes (FAOSTAT, 2008). Dans la caraïbe, le premier producteur est Haïti avec 353 000 tonnes produites en 2010. En Guadeloupe, l'igname constitue une des principales filières de diversification (6^{éme} rang des productions) avec 6900 tonnes produites en 2010. En Guadeloupe, cinq espèces sont consommées. *D. cayenensis-rotundata* pour ses qualités gustatives bien que sa durée de conservation après récolte soit courte. *D. alata* est moins appréciée pour ses qualités organoleptiques mais *a contrario* se conserve bien. *D. trifida* est l'espèce dont la chair est la plus appréciée cependant la production de cette espèce est limitée par les viroses. D'autres espèces sont consommées de façon mineure : *D. bulbifera* appelée localement « Adow » qui a une chair plutôt amer et *D. esculenta* appelé localement « pas possible » ou « cousse-couche douce » presque aussi appréciée que *D. trifida*.

La dioécie est une caractéristique de toutes les *Dioscorea* cependant des cas de monoécie ont été révélés (Burkill 1960). Alors que les espèces sauvages sont florifères, la plupart des ignames cultivées ont perdu leur capacité à fleurir depuis leur multiplication exclusivement végétative par fragments de tubercules ou mini-semences depuis leur domestication. La domestication des espèces *D. rotundata* et *D. cayenensis* est encore active en Afrique de l'Ouest où ces espèces sont cultivées dans le même habitat que les espèces sauvages apparentées, permettant le maintien de la diversité des espèces cultivées (Mignouna et Dansi 2003). L'espèce *Dioscorea alata* aurait été domestiquée vers 6000 BP, cependant elle n'est pas connue à l'état sauvage même dans son aire de diversification. La floraison et la

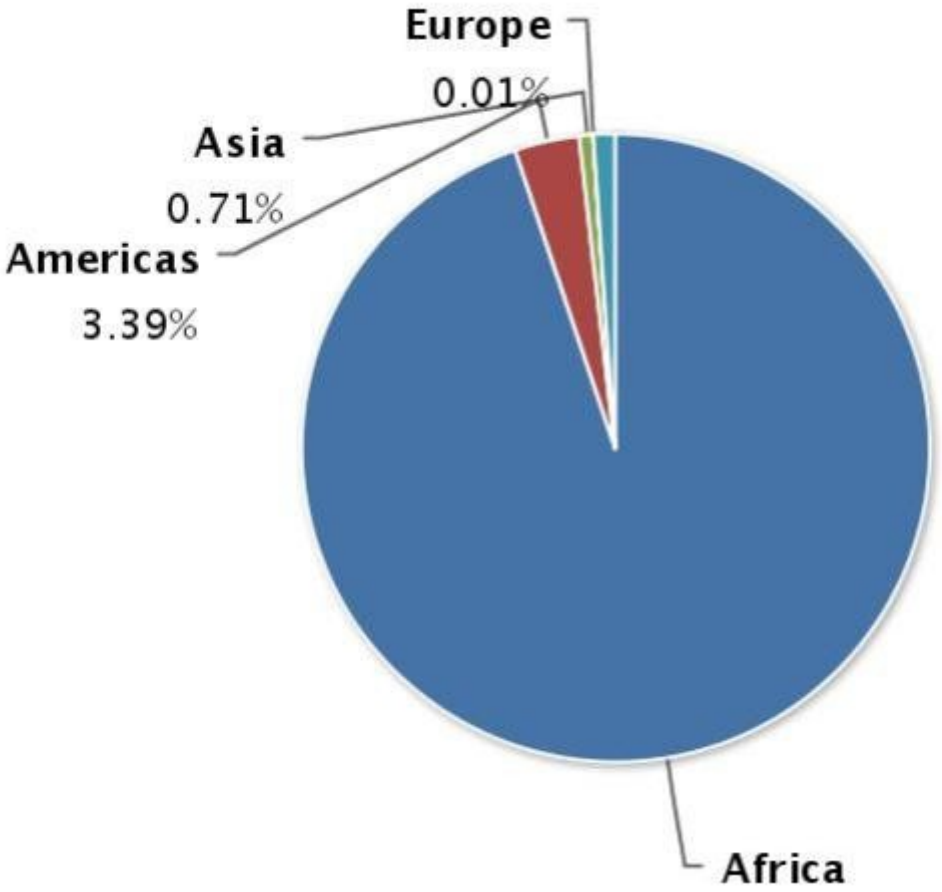

Figure 6 Production d'igname par région (moyenne de 1961-2010) d'après FAOSTAT, 2008.

fructification sont peu abondantes chez cette espèce comme chez l'espèce *D. rotundata* (Malapa et al. 2005).

L'espèce *D. alata* a longtemps été considérée comme étant stérile en raison de sa floraison erratique (Miège 1952, Coursey 1967) jusqu'à ce que la reproduction sexuée soit maîtrisée par Abraham pendant les années 1980 (Abraham et al.1986). Les pollinisations artificielles étaient réalisées à l'aide de la « pencil method » (Abraham et Nair 1990). Cette méthode consiste à prélever les étamines sur la fleur mâle à l'aide d'un crayon et de les déposer sur le stigmate de la fleur femelle.

Le fait que les fleurs mâles et femelles soient portées par des plants différents constitue une contrainte à la reproduction sexuée, nécessitant une dispersion du pollen par entomogamie, par les thrips (Segnou et al. 1992). Une autre contrainte à la reproduction sexuée est le fait qu'il existe un décalage entre la floraison des mâles et des femelles, les femelles fleurissant avant les mâles. La période de floraison est courte (environ trois mois) et les périodes de fertilité sont réduites. Chez les plantes femelles, les fleurs restent ouvertes de façon continue pendant une semaine, tandis que chez les mâles, l'ouverture des fleurs ne dure que 3 à 4 heures par jour. Pour une plante mâle donnée, la fertilité des fleurs s'échelonne sur une période de 15 - 25 jours.

L'inflorescence mâle est composée d'épis recouverts d'une dizaine de fleurs densément insérées, de petite taille (0,5 à 2 mm de diamètre) possédant six tépales et six étamines (figure 7). L'inflorescence femelle est composée d'épis de 15-25 cm de long, chaque épi porte dix à vingt fleurs de taille environ 10 fois supérieure à celle des fleurs mâles (figure 8). A l'époque de l'anthèse les fleurs femelles émettent un parfum. Les fruits sont des capsules rigides trilobées (figure 9), chaque lobe pouvant comporter deux graines. Entre une à six graines peuvent être obtenues après fécondation (Degras 1993).

La polyploïdie chez les plantes

La polyploïdie est la présence de plus de deux lots de chromosomes dans un organisme. Les organismes contenant 3, 4 ou 5 stocks chromosomiques sont appelés triploïdes, tétraploïdes et pentaploïdes respectivement. Alors que chez les animaux ce phénomène est le plus souvent létal, chez les plantes c'est un caractère commun. Soixante dix pour cents des angiospermes auraient connus au moins un épisode de polyploïdisation d'après Masterson (1994).

Figure 7 Inflorescence mâle de *D. alata*

Figure 8 Inflorescence femelle de *D. alata*

Figure 9 Fruits de *D. alata*

La polyploïdisation serait donc un élément important de l'évolution du génome des plantes (Soltis et Soltis 2009). Quelques-unes des cultures les plus importantes au monde sont polyploïdes telles que le blé, l'avoine, le coton, le tabac, la canne à sucre cultivée, la banane ou la pomme de terre.

Trois types majeurs de polyploïdes ont été définis par Stebbins (1950) sur la base de critères génétiques et cytogénétiques : les autopolyploïdes, les allopolyploïdes et les allopolyploïdes segmentaires. Les autopolyploïdes qui possèdent plusieurs copies du même génome sont caractérisés par la formation de multivalents à la méiose et un type de ségrégation polysomique (Annexe 2). Les allopolyploïdes sont issus de l'hybridation entre deux espèces différenciées suivie d'un doublement chromosomique. Ils sont caractérisés par des appariements sous forme de bivalents et une hérédité de type disomique et ressemblent donc aux diploïdes dans leur comportement cytogénétique. Les allopolyploïdes segmentaires sont issus de l'hybridation entre deux espèces proches et présentent un type de ségrégation intermédiaire du fait de la présence de lots de chromosomes homologues et de lots de chromosomes homéologues (Janoo et al. 2004 ; Stift et al. 2008).

Les espèces autopolyploïdes seraient plus nombreuses que ce qui est communément admis (Soltis et al. 2004). L'avantage majeur des autopolyploïdes est la vigueur hybride ou hétérosis (Gallais 2003, Parisod et al.2010). En effet puisqu'ils peuvent maintenir trois ou quatre allèles à un locus donné, ils auront une hétérozygotie supérieure à celle de leurs équivalents diploïdes, ce qui peut expliquer leur succès dans les populations naturelles dans lesquelles on observe en sympatrie les deux niveaux de ploïdie pour la même espèce.

Il existe deux modes de formation de polyploïdes dans les populations naturelles : le doublement somatique et l'union de gamètes non réduits. La formation de 2*n* gamètes a été observée chez de nombreuses espèces qui produisent des polyploïdes (Ramsey & Schemske, 1998). Il existe deux évènements cytologiques à l'origine des gamètes non réduits : la restitution de première division (First Division Restitution) et la restitution de seconde division (Second Division Restitution) (figure 10). Ces deux évènements ont des conséquences génétiques profondément différentes. Lors d'une FDR, tous les locus entre le centromère et le premier crossing-over qui sont hétérozygotes chez le parent diploïde seront hétérozygotes dans les gamètes. La moitié de ceux qui sont au-delà du crossing over seront

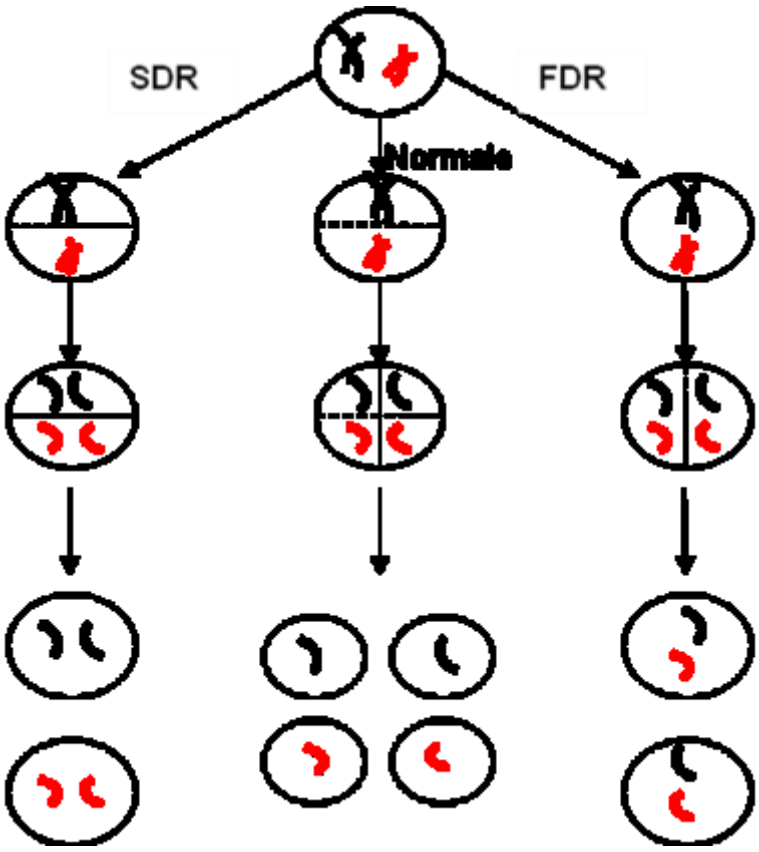

Figure 10 Schéma de formation de gamètes normaux n et de gamètes 2*n* de type SDR (Restitution de seconde division) et FDR (Restitution de première division) d'après Bretagnolle et Thompson, 1995.

hétérozygotes dans les gamètes. Lors d'une SDR tous les locus entre le centromère et le premier crossing over qui sont hétérozygotes chez le parent diploïde seront homozygotes dans les gamètes, tandis que ceux au-delà du crossing over seront hétérozygotes (Peloquin et al. 2008).

La FDR transmettrait donc une grande partie de l'hétérozygotie parentale à la descendance (100-50%), tandis que la SDR transmettrait 0-100% de l'hétérozygotie parentale en fonction de la position du locus vis-à-vis du centromère et de la présence d'un crossing over (Park et al. 2007).

La formation de polyploïdes *via* non réduction gamétique peut se réaliser :

- soit en une étape par l'union de deux gamètes non réduits comme c'est le cas chez l'espèce autotétraploïde *Dactylis glomerata* (Bretagnolle & Lumaret, 1995). Ce procédé est nommé « bilateral sexual polyploidisation » (BSP)

- soit en deux étapes impliquant un pont triploïde (triploid bridge). Ce procédé est nommé « unilateral sexual polyploidisation » (USP) et requiert la fertilité des triploïdes. Ce type de formation a été observé chez *Chamerion angustifolium* (Husband 2004), *Achilea borealis* (Ramsey 2007), *Cucumis sativus* (Diao et al.2009).

La polyploïdie chez *Dioscorea* spp.

La polyploïdie est importante dans le genre Dioscorea. La plupart des espèces cultivées sont polyploïdes avec des niveaux de ploïdie allant de 2*x* à plus de 10*x*. Une synthèse de l'ensemble des travaux effectués par comptage chromosomique sur les différentes espèces de Dioscorea est présentée dans Essad 1984. Toutefois du fait que les chromosomes d'igname sont de petites tailles 0,2-2µm (Ramachandran 1968) et ont tendance à s'agglutiner ces résultats doivent être relativisés. Par exemple, c'est le cas de l'espèce *D. alata,* qui a été longtemps considérée comme étant hautement polyploïde, comportant six niveaux de ploïdie (2*n*=30, 40, 50, 60, 70 et 80), alors que des études plus récentes ont prouvé que finalement elle ne comporte que trois cytotypes (2*n*= 40, 60, et 80) (Abraham et Nair 1991, Gamiette et al.1999, Malapa et al.2005, Arnau et al.2009).

Les nombres chromosomiques de base de ce genre étaient initialement considérés comme *x*=9,10 et 12 sur la base des études cytologiques publiées. Le nombre chromosomique de *x*=10 était reporté pour toutes les espèces asiatiques. Les espèces africaines et américaines étaient

considérées comme ayant un nombre chromosomique de 10 ou 9. Cependant, en 1984, Essad a démontré que le nombre chromosomique de base de l'espèce américaine *D.trifida* était *x*=10 et non pas *x*=9.

Des études plus récentes basées sur l'hérédité de marqueurs microsatellites ont remis en cause le nombre chromosomique de base des trois principales espèces cultivées : *D. rotundata* (Scarcelli *et al.* 2005), *D. trifida* (Bousalem *et al.* 2006) et *D. alata* (Arnau et al. 2009) qui auraient toutes *x*=20 chromosomes et non pas *x*=10.

Pour ce qui concerne *D. alata,* Arnau et al. ont démontré en 2009 que les variétés qui possèdent 40, 60, et 80 chromosomes sont respectivement diploïdes (2*n*=2*x*=40), triploïdes (2*n*=3*x*=60) et tétraploïdes (2*n*=4*x*=80) et non pas tétraploïdes, hexaploïdes et octoploïdes comme supposé. Cette espèce a été longtemps considérée comme étant autoallopolyploïde (Prain and Burkill 1939) issue de l'hybridation des espèces *D. persimilis* et *D. hamiltonii* (Mignouna et al.2002). Cependant la démonstration récente de la plus grande proximité génétique avec *D. numullaria* ou *D. transversa* que de *D. persimilis* (Malapa et al.2005) ainsi qu'une ségrégation de type diploïde des clones à 2*n* =40 (Arnau et al. 2009) a permis de remettre en cause l'origine autoallopolyploïde des *D. alata.*

L'amélioration génétique chez *D. alata*

Le premier programme d'amélioration chez *D. alata* a été initié en 1986 en Inde cependant le manque de connaissance concernant les origines, la diversité et la génétique de cette espèce a constitué une limite à l'efficacité des travaux d'amélioration.

Une étude menée par Abraham et Nair 1991 a permis de démontrer que le pollen des clones mâles du type 2*n*=40 chromosomes était très fertile, et que ces mâles montraient des méioses normales avec 20 bivalents. Les clones mâles à 2*n*= 80 chromosomes n'avaient pas été identifiés au sein de la collection. Pour les clones femelles trois cytotypes ont été observés à 2*n*=40 chromosomes, 2*n*= 60 chromosomes et 2*n*= 80 chromosomes. Seul le cytotype à 2*n*=40 chromosomes était capable de former des graines viables après pollinisation, les croisements intercytotypes entre femelles triploïdes et mâle diploïdes ou entre femelles tétraploïdes et mâles diploïdes ne produisant aucunes graines. Dans cette même étude Abraham a conclu en 1991 à la stérilité des triploïdes. En effet, les boutons floraux des mâles restent fermés jusqu'à ce qu'ils se dessèchent et les croisements entre femelles triploïdes et mâles diploïdes n'ont pas permis d'obtenir de graines viables.

Jusqu'à 2006, les programmes d'amélioration ont été basés sur des croisements entre variétés diploïdes, cependant des études récentes ont mis en évidence que chez l'igname aussi l'augmentation de la ploïdie est corrélée à une augmentation de la vigueur, à des rendements plus élevés et plus stables et à une augmentation de la tolérance aux stress biotiques et abiotiques (Malapa et al. 2005; Lebot 2009; Arnau et al. 2010). En conséquence le programme d'amélioration développé sur l'espèce *D. alata* au CIRAD Guadeloupe depuis 2006 a été orienté vers la création de variétés polyploïdes (tri et tétraploïdes). Ces variétés présentent un potentiel de rendement supérieur même dans des sols appauvris, une meilleure vigueur au champ, une bonne couverture du billon et une tolérance à l'anthracnose causée par le champignon *Colletotrichum glosporoïdes*. Ces propriétés pourraient permettre de limiter l'apport d'intrants et d'herbicides et d'éviter l'utilisation de pesticides en sachant qu'ils sont dommageables pour l'environnement et la préservation des écosystèmes.

Des accessions tétraploïdes distantes génétiquement ont été sélectionnées et croisées pour produire des descendances tétraploïdes et maximiser l'hétérozygotie (figure 11). Par ailleurs, des diploïdes doublés par un agent chimique (colchicine) ont été produits à partir de variétés élite diploïdes pour pouvoir les utiliser en tant que géniteurs tétraploïdes. Des croisements entre variétés di et tétraploïdes ont également été réalisés afin d'obtenir des descendances triploïdes ou tétraploïdes par formation de gamètes non réduits. Une synthèse sur les croisements aboutissant à la production de polyploïdes est présentée dans le tableau 1. Cependant il n'existe pas de connaissances chez *D. alata* sur le type de ségrégation des variétés tétraploïdes, ni sur les phénomènes de diplogamétisation, ni sur les phénomènes d'incompatibilité au niveau de l'albumen conduisant à la stérilité de certains croisements.

L'utilisation des marqueurs moléculaires notamment les marqueurs microsatellites pourrait permettre d'accélérer l'acquisition de ces connaissances. A ce jour 214 marqueurs sont disponibles dont 55 marqueurs ont été développés à partir de sept espèces différentes de Dioscorea : *D. tokoro, rotundata, abyssinica, praehensilis, japonica, trifida et alata* (Misuki et al.2005, Tostain et al.2006). De plus, un projet commun INRA-CIRAD a permis le développement de 159 marqueurs supplémentaires, 69 développés sur l'espèce *D. alata* et 90 développés sur l'espèce *D. rotundata* (Andris et al. 2010).

Tableau 1 : Synthèse des connaissances sur les types de croisements aboutissant à la production de polyploïdes

Croisement		Descendance attendue	Ploïdie de l'albumen attendue	Ratio GM/GP	Descendance obtenue
Femelle	Mâle				
2*x*(*n*)	2*x*(*n*)	2*x*	3*n*	2: 1	2*x*
2*x*(2*n*)	2*x*(*n*)	3*x*	5*n*	4: 1	3*x*
2*x*(*n*)	2*x*(2*n*)	3*x*	4*n*	2: 2	3*x* SEI
2*x*(2*n*)	2*x*(2*n*)	4*x*	6*n*	2 :1	-
2*x*(*n*)	4*x*	3*x*	4*n*	2: 2	3*x* SEI
2*x*(2*n*)	4*x*	4*x*	6*n*	2: 1	4*x*
4*x*	2*x*(*n*)	3*x*	5*n*	4 :1	rien
4*x*	2*x*(2*n*)	4*x*	6*n*	2 :1	-
3*x*(2*n*)	2*x*(2*n*)	4*x*	6*n*	2 :1	rien
3*x*(2*n*)	4*x*	4*x*	6*n*	2 :1	rien
4*x*	4*x*	4*x*	6*n*	2 :1	4X

Les mâles triploïdes sont stériles car leurs boutons floraux restent fermés jusqu'à dessèchement. GM/GP : génome maternel/ génome paternel ; S.E.I: sauvetage d'embryons immatures

Figure 11 Pollinisation artificielle d'une femelle tétraploïde à l'aide de la « pencil method »

Manipulation de la ploïdie du gamétophyte, de l'albumen et du sporophyte pour l'amélioration : avancées chez *D. alata* par comparaison avec le modèle de la pomme de terre.

Chez la pomme de terre, la manipulation de la ploïdie à l'aide d'haploïdes, de 2*n* gamètes et d'espèces sauvages a été la méthode d'amélioration découlant de recherches cytogénétiques, la plus innovante (Ortiz et al.2005).

Les haploïdes (2*x*) de *Solanum tuberosum* (4*x*) sont utilisés pour incorporer les gènes d'intérêts des ressources génétiques des espèces sauvages diploïdes dans la forme cultivée (Janski et al.1990). Les haploïdes sont des individus qui ont le même nombre de chromosomes que les gamètes. Les descendants diploïdes sélectionnés peuvent être transférés au niveau tétraploïde par polyploidïsation sexuelle en utilisant les 2*n* gamètes.

Chez la pomme de terre la manipulation de la ploïdie via les 2*n* gamètes a été initiée en 1967. Des croisements intercytotypes (inter ploïdies) ♀4*x* × ♂2*x* ont permis de mettre en évidence la formation de 2*n* gamètes. En effet peu ou pas de descendances triploïdes ont été obtenues lors de ces croisements par contre des descendants tétraploïdes ont été obtenus et leur nombre était dépendant du génotype diploïde (Hanneman and Peloquin 1967). La capacité à produire des descendances 4*x* lors de croisements ♀4*x* × ♂2*x* résulte de la production de 2*n* pollen à des pourcentages variables. D'autres recherches chez la pomme de terre ont permis d'identifier l'implication de trois mutants dans la diplogamétisation et de distinguer les mécanismes et les modes de formation des 2*n* gamètes. Le mutant le plus fréquent, « parallel spindles » *ps*, affectant l'orientation des fuseaux à l'Anaphase II et génétiquement équivalent à une FDR, est sous le contrôle d'un allèle récessif *ps* (Mok et Peloquin 1975). Deux autres mutants moins fréquents « premature cytokenesis » -1 et -2 sous le contrôle génétique des allèles récessifs *pc-1* et *pc-2* sont équivalents à une SDR (Mok et Peloquin 1975).

Les croisements intercytotypes ♀2*x* × ♂4*x* effectués chez la pomme de terre (Hanneman et Peloquin 1968) ont permis de mettre en évidence la formation de 2*n* mégagamétophytes. Par la suite Stelly et Peloquin 1986 ont démontré que ces 2*n* mégagamétophytes provenaient le plus fréquemment de l'omission de la seconde division, contrôlée par un mutant récessive *os* équivalent à une SDR (Werner et Peloquin 1991).

Par ailleurs chez cette espèce comme chez d'autres les hybrides triploïdes sont rares lors de croisements ♀4*x* × ♂2*x*, ♀2*x* × ♂4*x* ou ♀2*x* × ♂2*x* (Hanemann et Peloquin 1968).

Chez *D. alata* l'utilisation des ressources sauvages dans les programmes d'amélioration comme c'est le cas chez la pomme de terre n'est pas possible puisque l'espèce sauvage n'a pas été identifiée.

Les croisements intercytotypes ♀2*x* × ♂4*x* ont permis d'obtenir des descendances triploïdes via le sauvetage d'embryons immatures et quelques individus tétraploïdes non attendus ont été observés (Arnau et al. 2006). Les croisements intercytotypes (♀4*x* × ♂2*x*) ont été réalisés cependant aucune descendance n'a été obtenue. Il y a eu gonflement de fruits dépourvus de graines. De plus de rares triploïdes spontanés ont été observés lors de croisements 2*x* × 2*x* (Arnau et al. 2006).

L'obtention de triploïdes au sein de descendances 2*x* × 2*x* et de tétraploïdes au sein de descendances 2*x* × 4*x* a permis de suggérer que la formation de gamètes non réduits serait à l'origine des phénomènes de polyploidïsation chez *D. alata*. Cependant cette origine probable n'a jusqu'alors pas été démontrée et les mécanismes impliqués dans la formation des polyploïdes sont à élucider.

L'albumen est spécifique des angiospermes, il se développe après double fertilisation, où un gamète mâle va féconder l'oosphère pour former le zygote tandis que l'autre va féconder la cellule centrale, elle-même constituée de deux noyaux polaires pour former l'albumen. L'albumen est triploïde avec un ratio génome maternel sur génome paternel de 2 :1. Le développement normal de la graine *in vivo* est conditionné par l'albumen. En effet lors de croisements intercytotypes, le développement anormal de l'albumen a fréquemment pour conséquence la non viabilité des graines. Chez la plupart des angiospermes les croisements intercytotypes ne produisent pas de descendances triploïdes viables, phénomène appelé le bloc triploïde « triploid block » (Marks 1966). Le ratio 2 :1 génome maternel par rapport au génome paternel est important car toutes déviations de ce ratio produit un albumen défectueux et une graine non viable. Par exemple lors de croisements 4*x* × 2*x* et 2*x* × 4*x*, le développement de l'albumen est anormal car le ratio femelle-mâle est 4:1 et 1 :1 respectivement. Cependant s'il y a formation de 2n gamètes chez le parent diploïde le ratio est 2 :1 et le développement de l'albumen est normal. Ainsi les barrières post-zygotiques dues à des incompatibilités au niveau de l'albumen peuvent être surmontées par les 2*n* gamétophytes (Carputo et al.1997).

La méthode d'amélioration impliquant la manipulation de la ploïdie à l'aide des 2*n* gamètes en tenant compte des incompatibilités au niveau de l'albumen a été étendue au manioc, à la patate douce, à la banane, à la luzerne et à d'autres plantes d'importance économique (Ortiz 2009).

OBJECTIFS DE L'ETUDE

L'objectif général est d'étudier la polyploidïsation chez *D. alata* dans le but d'optimiser les stratégies de création d'hybrides polyploïdes et hétérozygotes.

Les objectifs spécifiques de ces travaux visent à :

1/ Déterminer la nature auto ou allotétraploïde des cytotytpes de *D. alata* à 80 chromosomes

Nous avons utilisé des marqueurs microsatellites pour définir le type d'hérédité en appliquant une approche Bayésienne dans une population en ségrégation (Partie1.1). Cette connaissance est nécessaire pour améliorer les schémas de sélection à ce niveau de ploïdie.

2/ Produire des connaissances cytologiques sur les appariements chromosomiques se réalisant au sein des méioses des cytotypes à 80 chromosomes

Nous avons étudié les méioses de clones mâles $2n$=80 et mené des études de fertilité des femelles et des mâles tétraploïdes dans le but de déterminer la nature de la ploïdie de ces clones (Partie 1.2).

3/ Comprendre l'origine des hybrides $3x$ et $4x$ spontanément obtenus lors de croisements intracytotypes et intercytotypes

En utilisant la cytométrie en flux et les marqueurs microsatellites sur les individus tirés de différentes populations en ségrégations, obtenues en croisant des géniteurs diploïdes connus pour leurs aptitudes à produire des polyploïdes. La cytométrie en flux a été également utilisée pour mesurer les niveaux de ploïdie des albumens afin de comprendre le rôle des barrières dues au bloc triploïde. (Partie 2).

Ces trois chapitres sont présentés sous forme de publications, deux sont acceptées (Partie 1), la troisième est soumise.

PARTIE 1 : TYPE DE SEGREGATION DES VARIETES TETRAPLOIDES

1.1. Evidence génétique

Cette partie est présentée sous forme d'article : « Inheritance pattern of tetraploid *Dioscorea alata* and evidence of double reduction using microsatellite marker segregation analysis » accepté dans *Molecular Breeding*.

Résumé

Des études récentes ont montré que le nombre chromosomique de base des trois principales espèces d'ignames cultivées *Dioscorea alata*, *D. rotundata* and *D. trifida* est $x = 20$, et que les clones avec $2n = 40$ chromosomes sont diploïdes. Les programmes d'amélioration de *D. alata* étaient limités à la production d'hybrides diploïdes, jusqu'en 2006 où la fertilité des tétraploïdes ($2n = 80$) a été découverte et que les premiers hybrides ont été produits par hybridation manuelle. Cependant, le type de polyploïdie (autotétraploïdie ou allotétraploïdie) des clones tétraploïdes de *D. alata* n'était pas connu. Dans cette étude, les patrons d'hérédité de marqueurs microsatellites ont été déterminés dans une descendance tétraploïde en utilisant une approche Bayésienne et en recherchant des évènements de double réduction. Les résultats obtenus confirment la nature autotétraploïde des clones $2n = 80$ de *D. alata*.

Mots clés: Igname; *Dioscorea alata;* Polyploïdie; ségrégation polysomique

1.1.2. Article I

Inheritance pattern of tetraploid *Dioscorea alata* and evidence of double reduction using microsatellite marker segregation analysis

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Molecular Breeding, DOI: 10.1007/s11032-012-9749-0

accepté le 3/5/ 2012

Abstract

Recent studies have shown that the basic chromosome number of the three major edible yams, *Dioscorea alata*, *D. rotundata* and *D. trifida,* is x = 20, and that the clones with 2n = 40 chromosomes are diploids. *D. alata* breeding programmes were limited to the production of diploid hybrids until 2006, when the tetraploids (2n = 80) were found to be fertile and polyploid hybrids were produced by conventional hybridisation. However, the nature of polyploidy was not known in *D. alata* tetraploid clones (autotetraploidy or allotetraploidy). In the present study, the inheritance pattern of SSR markers was determined in a tetraploid progeny using a Bayesian approach and by examining double reduction events. Results obtained confirm the autotetraploid nature of the 2n = 80 clones of *D. alata*.

Keywords: Yam; *Dioscorea alata;* Polyploidy; Polysomic inheritance.

Introduction

The genus *Dioscorea* includes over 600 species among which the Asian species *D. alata*, the African species *D. cayensis-rotundata* and the American species *D. trifida* are the major species cultivated. For *D. rotundata* and *D. trifida*, two recent reports have completely reformed the knowledge about their ploidy levels. *D. rotundata,* assumed to be tetraploid, is in fact diploid (Scarcelli et al. 2005), and *D. trifida,* previously thought to be octoploid, becomes the first autotetraploid species identified in the genus *Dioscorea* (Bousalem et al. 2006). *D. alata is* the most widespread yam worldwide and includes accessions with $2n = 40$, 60 and 80 chromosomes. A recent report based on flow cytometry, chromosome counting and microsatellite segregation analysis has demonstrated that *D. alata* accessions with $2n = 40$, 60 and 80 chromosomes are diploid, triploid and tetraploid ($2n = 2x$, $3x$, $4x$), respectively, and not tetraploid, hexaploid and octoploid, as previously assumed (Arnau et al. 2009). Consequently, the three studies referred to above provided the genetic evidence that the basic chromosome number of these three species is x = 20 and not x = 10, as previously believed.

The importance of yams in terms of food security has given impetus to the establishment of several genetic improvement programmes of *D. alata* throughout tropical regions in order to develop new varieties that combine the traits of high and stable tuber yield, good tuber quality and resistance to abiotic and biotic stresses. Ploidy increase in *D. alata* is correlated with growth vigour, higher tuber yield and increased tolerance to abiotic and biotic stress (Malapa et al. 2005; Lebot 2009, Arnau et al. 2010). Although polyploidy has been recognised for a long time in *D. alata* (Abraham and Nair 1991), all breeding programmes were based on the creation of diploid varieties until 2006. The first polyploid hybrids [triploid $2n = 3x = 60$ from ($2n = 2x = 40$) × ($2n = 4\ x = 80$) crosses, and tetraploid $2n = 4x = 80$ from $2n = 4x = 80 \times 2n = 4x = 80$] were recently created by conventional hybridisation, thanks to the discovery of the fertility of *D. alata* tetraploid varieties and to the development of an *in vitro* immature embryo rescue method (Arnau et al. 2010). However, no studies were previously undertaken to elucidate the type of polyploidy (allo or autopolyploidy) present in 2n = 80 tetraploid *D. alata* varieties.

Allopolyploidy is considered the most common mode of polyploidy speciation in angiosperms (Grant 1981). Although autopolyploidy is believed to be less common than allopolyploidy, recent tetrasomic inheritance data have demonstrated an autopolyploid origin for an increasing number of polyploids (Lumaret and Borril 1988; Rieseberg and Doyle 1989; Soltis and Soltis 1989; Wolf et al. 1990; Jackson and Jackson 1996; Segraves et al. 1999; Bousalem et al. 2006; Landergott et al. 2006). Allopolyploidy is thought to result from the

chromosome combination of distinct genomes and subsequent chromosome doubling and disomic inheritance, while autopolyploidy is assumed to result from the chromosome doubling of genetically similar genomes, primarily by fusion of unreduced gametes (Stebbins 1950; Bretagnolle and Thompson 1995), resulting in tetrasomic inheritance.

Disomic inheritance of alleles is characteristic of allotetraploids because the homeologous chromosomes are sufficiently different that they cannot pair during meiosis, and one chromosome from each set always segregates into each gamete. In contrast, segregation patterns in autopolyploids are more complex because completely homologous chromosomes have equal chances to pair at meiosis, leading to either bivalents or multivalents (mostly tetravalents).

Intermediate inheritance may also be expected in interspecific hybrids formed from the hybridisation of two closely related species since they may have some identical homologous chromosome sets and some dissimilar homeologous chromosome sets (Jannoo et al. 2004; Stift et al. 2008). In general, three types of information are used to discriminate between the different types of inheritance patterns of tetraploids: segregation patterns of genetic markers, the presence or absence of multivalent formation at Metaphase I of meiosis, and regeneration of tetraploids from the parental diploids (Stebbins 1950). Even if multivalent formation is more common in autotetraploid species than in allotetraploid species, counting multivalents is, however, not a completely reliable method since autotetraploid species do not always exhibit multivalent formation (Soltis and Riesberg 1986) and allotetraploid species have been shown to form multivalents at times (Watson et al. 1991).

In a segregating population obtained from the cross between two tetraploid varieties with genotypes abcd and efgh, respectively, at a given locus, 36 genotypes would be expected in the case of autopolyploidy since six gametes would be formed in equal proportion in each parent (ab, ac, ad, bc, bd, cd, and ef, eg, eh, fg, fh, gh, respectively).

In the case of allopolyploidy only 16 genotypes would be expected since preferential pairing between homologous alleles would reduce the possible number of gametes. Considering that in female, a and b alleles are carried by the same homologous chromosome set and the other two alleles (c and d) by the other set, and that in male, e and f alleles are carried by the same homologous chromosome set and the other alleles (g and h) by the other set, a total of four gametes would be formed in equal proportion in each parent (ac, ad, bc, bd and eg, eh, fg, fh, respectively), and some genotypic classes would be absent in the progeny compared to autopolyploidy.

If the two parents have four different alleles, the parental genotypes and the genotypes

expected in the progeny are directly deduced from the observed phenotype. When the parents have less than four alleles, several parental genotypes that correspond to different associations of alleles are possible. For example, a genitor with a phenotype ab could be aabb (duplex) or aaab or abbb (one simplex + one triplex), or aab0 or abb0, considering one null allele. A Bayesian approach is more appropriate for testing complex segregation patterns than χ^2 statistics because it allows testing several hypotheses at the same time. It incorporates a multinomial distribution to determine the probabilities of observing the progeny genotypes under each hypothesis or likelihood. This approach is able to assess the confidence in a particular hypothesis relative to the entire set of hypotheses being considered (Olson 1997).

If a tetraploid variety is revealed to be autotetraploid, then tetravalents could be formed at meiosis and double reduction events could also be observed. Double reduction can occur if tetravalents are formed at Metaphase I of meiosis, if a crossover occurs between a locus and the centromere and if the two sister chromatids migrate to the same pole in Anaphase II of meiosis. The coefficient of double reduction depends on the position of the locus in relation to the centromere (Mather 1936; Fisher 1947). In a cross between two varieties with four different alleles each (abcd X efgh), six heterozygote gametes would be expected for each parent (ab, ac, ad, bc, bd, cd, and ef, eg, eh, fg, fh, gh, respectively) leading to a progeny in which all individuals would carry four different alleles. The presence of individuals with three or two alleles would be a consequence of double reduction in one or both genitors, leading to unexpected homozygote gametes (aa, bb, cc, dd, and ee, ff, gg, hh, respectively). Whereas 36 genotypes are expected in the absence of double reduction, 100 genotypes can be obtained in the presence of double reduction since ten gametes can be formed at meiosis, six heterozygote and four homozygote resulting from double reduction. Double reduction effects are assumed to reduce heterozygosity in segregated populations. Since double reduction is one of the distinctive features of tetrasomic inheritance (Ronfort et al. 1998; Luo et al. 2000; Wu, Gallo-Meagher et al. 2001; Wu, Wu et al. 2001; Luo et al. 2006), the observation of this phenomenon in a segregating population should be an indication of meiosis matching type.

The aim of the present study was to use microsatellite markers to determine if the inheritance pattern of tetraploid *D. alata* is autopolyploid or allopolyploid by applying a Bayesian approach to describe the probabilities of all the possible genotypes in a segregating population under the two inheritance patterns and by examining double reduction events.

Materiels and methods

Plant materials for segregation analysis

A progeny of 188 individuals obtained by crosses between two natural tetraploid parents (Noulelcae x Tepuna), genetically distant were analysed. Both progenitors are originated from Vanuatu, one of the diversification centres of the yam, *D. alata* (Malapa et al. 2005). Controlled crosses were done by manual hybridisation.

Ploidy level: Flow cytometry analysis

It has been observed that the female Noulelcae is able to produce unreduced gametes (Nemorin et al., in preparation), which can take part in fertilisation, producing individuals with higher ploidy levels than the parental plants. The 188 individuals were analysed by flow cytometry to check their ploidy level.

Flow cytometry was performed on nuclei solutions obtained from fresh leaf samples, as described by Arnau et al. (2009). Nuclei were stained with propidium iodure, and DNA quantities were measured by a FACScalibur Laser flow cytometer (Beckton Dickinson, USA).

DNA extraction

Nuclei were first extracted from 100 mg of fresh leaves by a specific triton buffer using the method described by Arnau et al. (2002). DNA was then extracted according to the instructions in the DNeasy Plant Mini Kit (Qiagen).

Microsatellite amplification

We used 20 SSR markers selected from a set of 98 SSRs developed from five different yam species: *D. rotundata*, *D. abyssinica*, *D. praehensilis*, *D. japonica* and *D. alata* (Misuki et al. 2005; Tostain et al. 2006; Andris et al. 2010).

Amplification was performed in a total volume of 20 µL containing 0.05 U/µL of polymerase Taq, 2 µl of 10X buffer, 0.2 mM of dNTP, 0.2 µmol of each primer, 2 mM of $MgCl_2$ and 10 ng of DNA. Forward primers were labelled with one of the following fluorophores: TET, NED, HEX or 6-FAM. PCR conditions were as follows: 5 min of denaturation at 94°C, followed by 30 cycles alternating 30 sec of denaturation at 94°C, 30 sec of hybridisation at annealing temperature, 35 sec of extension at 72°C, and ending with 5

min of final elongation at 72°C. PCR was carried out using a PTC100 thermocycler (MJ Research).

Electrophoregrams were obtained by analysis of amplification products on an ABI PRISM-TM 3100 automatic sequencer (Applied Biosystems), and segregation profiles were analysed using GeneMapper v3.7 software (Applied Biosystems).

Microsatellite segregation analysis

Segregation pattern

A Bayesian method was used to discriminate among the different transmission hypotheses (auto or allotetraploidy). The probabilities of observing the progeny genotypes under each hypothesis or likelihoods were computed using the multinomial distribution described by Olson (1997):

$$P_r\,(\text{data}—H_j) = \frac{n!}{n_1!n_i!..n_k!}(P_i(H_j))^{n_1}\,(P_i(H_j))^{n_i}..(P_k(H_j))^{n_k}$$

where n is the total number of progeny, n_i is the observed number of progeny of genotype i, and $P_i(H_j)$ is the expected proportion of genotype i under hypothesis j. To take the observed, but unexpected genotypes under a given scenario into account, a P error value of 10^{-3} was assigned to this genotypic class (Bousalem et al. 2006). For the two hypotheses, the expected proportions of each genotype in progeny and likelihoods were computed using a PERL program designed by J. David (Bousalem et al. 2006).

We computed a Bayes factor to compare allo vs auto tetraploidy.

$$BF = \frac{\Pr\ (data \mid autotetraploidy)}{\Pr\ (data \mid allotetraploidy)} = \frac{\Pr\ (data \mid Hd_1) + \Pr\ (data \mid Hd_2) + \ldots + \Pr\ (data \mid Hd_i)}{\Pr\ (data \mid Ht_1) + \Pr\ (data \mid Ht_2) + \ldots + \Pr\ (data \mid Ht_i)}$$

where Hd_i is the *ith* possible parental genotype under the autotetraploid hypothesis, and Ht_i is the *ith* possible parental genotype under the allotetraploid hypothesis. A Bayes factor > 200 indicates that the autotetraploidy hypothesis is 200 times more likely than the allotetraploidy hypothesis, and provides very strong evidence to support the autotetraploid hypothesis (Kass and Raftery 1995; Bousalem et al. 2006).

When less than 4 alleles were observed at a given locus in parental genotypes, all allelic dosages and the possibility of one null allele were examined.

For allopolyploidy, Bayesian probabilities were calculated under the homoplasy hypothesis (i.e., two independently segregating loci can present alleles of the same size; Estoup et al. 1995). For example, a parent can be a/b at the locus in one genome and a/c in the second genome. This assumption is very conservative for testing inheritance patterns but leads to a lower discrimination between allotetraploid and autotetraploid patterns. A no homoplasy

hypothesis (i.e., alleles of the same size could not be shared between the two loci) was also tested.

Moreover, the MAC-PR method (microsatellite DNA allelic counting-peak ratios; Esselink et al. 2004) was used to determine the allelic dosage of two parental genotypes (Arnau et al., in prep) and precise allelic configurations could be assigned to two parental genotypes for almost all of the loci investigated. These dosage ratios were compared to the likelihood of the different possible parental genotypes computed from the segregation data.

Double reduction analysis

All loci that allowed discrimination between the two tested hypotheses (auto or allotetraploidy) were studied for the phenomenon of double reduction. The double reduction coefficient and its significance were calculated using TetraploidMap Software (Hackett and Luo 2003). Goodness of fit of the parental genotypes and significance of the double reduction coefficient alpha have been determined by a chi-square test (Luo et al. 2000)

Genetic linkage analysis

Genetic linkage analyses were performed to check whether the SSR loci tested represented an independent set of markers in *D. alata* genome. Independence of the SSR loci was tested using the routine TWOPOINT of TetraploidMap Software (Hackett and Luo 2003). Recombination frequencies and LOD scores between every pair of markers were calculated using the EM algorithm, as described by Luo et al. 2001.

Results

Flow cytometry

The results of flow cytometric analysis showed only individuals with DNA quantity compatible with $2n = 80$ chromosomes and none compatible with 2n=120 or 160. All 188 individuals were thus kept for further analysis.

Population screening for apomixis and contaminating genotypes

The possibility that some individuals had a different male progenitor than Tepuna or were the result of apomictic reproduction was verified. Two individuals showing only maternal alleles at all loci, which are in accordance with a phenomenon of apomictic reproduction, were identified. These two individuals were excluded from analyses.

Microsatellite screening for segregation analysis

Only the SSR markers that revealed a minimum of three total alleles on the two parents were selected for the segregation analysis. In a recent study, Bousalem et al. (2006) showed that

highly polymorphic markers are particularly useful to distinguish between the disomic and tetrasomic inheritance hypotheses. A screening of the two parents allowed to select a total of 20 markers among the 98 SSR tested, three of which showed a total of five alleles, nine showed a total of four alleles and eight showed three alleles. The remaining SSRs were not retained either because they did not reveal polymorphism between the two parents or because they revealed only two alleles.

Autopolyploidy versus allopolyploidy

To illustrate the computation of the Bayes factor, the results obtained for the locus mDaCIR108 are shown in Table 1. The female Noulelcae has three alleles (abd) and the male Tepuna has three alleles (phenotype bcd). Since both genitors have only three alleles, they could be either duplex for one of the 3 alleles or one allele could be null. This yields 16 scenarios for the 4x inheritance (in absence of double reduction) and 576 scenarios for the 2x inheritance pattern. The likelihoods of all these scenarios were calculated according to the data.

The Bayes factor (BF) testing the ratio of the sum of likelihood of all 4x scenarios on the sum of all 2x scenarios clearly indicates that the tetrasomic inheritance is much more likely than the disomic inheritance. The tetrasomic inheritance is 10^5 times more likely than the disomic inheritance when considering homoplasy (BF= 10^5). Under the most restricting conditions (non-shared allelic size), the Bayes factor in favour of 4x inheritance is even higher (BF = 10^{25}).

Among the likelihoods of the 16 scenarios for the 4x inheritance (in absence of double reduction), the most likely parental genotypes are “abd0” for the female and “bcdd” for the male. The MAC-PR method predicted the same genotypes.

The Bayes factors obtained for the 20 analysed SSRs are given in Table 2. All three loci with five segregating alleles provided very strong evidence in favour of the autopolyploid inheritance (Bayes factor: DIJ0443 = 10^{42}, DIJ0342 = 10^{37}, Dpr3B12 = 10^{17}). The patterns of seven of the nine loci with four segregating alleles also support a polysomic inheritance with Bayes factors > 200 (mDaCIR66 = 2.9 10^2, mDaCIR179 = 1.6 10^3, Dab2D07 = 1.6 10^4, mDaCIR51 = 3.8 10^5, mDaCIR108 = 4.6 10^5, mDaCIR8 = 4.1 10^9, Dab2D11 = 2.3 10^{14}). Under the homoplasy hypothesis, no statistical difference clearly appeared between the likelihood of 4x and that of 2x for the other two loci with four segregating alleles, and for all eight loci with three segregating alleles. In one case only, the 2x inheritance pattern appeared slightly more likely than the 4x scenario (locus Dpr3E10 with a Bayes factor = 3.14 10^{-2}, However under the most restricting conditions (non-shared

allelic size) the likelihoods of the 4x inheritance were much higher that the 2x inheritance for all 20 loci (10^2<BF<10^{93}).

Testing independence of loci

Linkage analyses could be done for all loci using the Two Point test of TetraploidMap Software. Results obtained are in accordance with a link between DIJ0443 and DIJ0342 (recombination frequencies= fr = 0.071, LOD 61), Dab2D11 and mDaCIR108 (fr= 0.3, LOD 5), Dpr3B12 and mDaCIR66 (fr= 0.22 LOD 9), Dab2E07 and Da1F08 (fr= 0.05, LOD 4).

Calculation of double reduction coefficient

Double reduction analyses were performed on the 20 loci (Table 3). This table gives the parental genotypes expected to be observed in presence of double reduction.The value of the double reduction coefficient is significant and maximal (α= 0.16) for three loci: Dpr3E10, Da2F10 and mDaCIR8, thirteen other loci revealed intermediate values of α The remaining four loci did not give a significant double reduction coefficient. Some individuals with unexpected phenotypes were observed for most of the loci (Table 3). These phenotypes can only be explained by double reduction events. For some loci with significant alpha values, no unexpected individual was observed. For these loci, the individuals resulting from the double reduction correspond to expected phenotypic classes, leading to a distortion in comparison to a model with no double reduction. For linked loci with significant value of double reduction, levels of double reduction were compared. Loci DIJ0443 and DIJ0342 showed similar value of double reduction (0.05 and 0.04). On the same manner Dab2D11 and mDaCIR108 showed similar value (0.03 and 0.05).

Discussion

The main difficulty encountered in genotyping polyploids is allelic dosage. The MAC-PR technique (microsatellite DNA allelic counting-peak ratios) was developed by Essenlink (2004) to assign allelic configurations in polyploids. This approach has been used successfully in several tetraploid species such as rose and citrus (Babaei et al. 2007; Helsen et al. 2009; Ferrante et al. 2010). This method was used to determine the allelic dosage of both progenitors of this study (Arnau et al., in prep). In this study, all possible parental genotypes were tested using the Bayesian procedure. Results obtained confirmed a correspondence between the parental genotype directly determined with allelic dosage using MAC-PR, and

the most likely genotypes predicted on segregation data using the Bayesian method and TetraploidMap Software.

We have observed that loci with four or five segregating alleles have the best discriminating power that is in accordance with results obtained on *D. trifida* (Bousalem et al. 2006). An explanation could be that the greater the number of alleles is, the larger the number of genotypic classes that could discriminate the two hypotheses will be.

Bayes factors obtained in this study for most polymorphic SSR (four-five segregating alleles) discriminate better than those obtained by Bousalem et al. (2006) to determine autopolyploidy in *Dioscorea trifida*. This can be explained by the larger population size in our study (n=188 vs n=60). The importance of segregation population size to differentiate between the 4x and 2x inheritances has already been underlined by Bousalem et al. (2006).

Despite the fact that hypotheses of octoploidy versus tetraploidy were not specifically tested in this study, our results confirmed those of Arnau et al. (2009) that showed that varieties with 80 chromosomes are tetraploid. Indeed, no individuals carrying more than four alleles were observed in the progeny for any of the three loci with five segregating alleles tested.

The results presented in this study show that tetraploid *D. alata* varieties have a clear polysomic inheritance pattern and are therefore autotetraploid. All 20 loci analysed showed Bayes factors in favour of autotetraploidy when considering a mutation model with no homoplasy. When assuming homoplasy (shared allelic size at homologous loci under the allotetraploid scenario), only loci with four and five segregating alleles made it possible to discriminate between the two segregation hypotheses since the remaining loci have no statistical power to discriminate the two inheritance patterns.

Segregation data and appropriate Bayesian approach confirmed their power in detecting chromosomic inheritance, as already demonstrated for another major species: e.g., the autotetraploidy in *D. trifida* (Bousalem et al. 2006), or disomy in two *Borderea* species (Catalán et al. 2006).

If segmental allopolyploidy has been observed in some species, e.g., in complex sugarcane hybrids (Janoo et al. 2004) segmental allotetraploidy seems unlikely in our case. Indeed, none of the 20 studied loci here gave a significant result in favour of allotetraploidy. It is still possible that unmarked chromosomes may show some homeologous pairing. This remains to be investigated by increasing the number of locus.

Autotetraploidy is characterised by the occurrence of double reduction events that lead to new homozygote genotypes and that reduce heterozygosity. The observation of

double reduction in 16 of the 20 loci analysed is consistent with a tetrasomic inheritance. Double reduction causes systematic segregation distortion and also a more complicated distribution of offspring genotypes in autotetraploid species (Luo et al.2006). Distortion segregation observed in our population can be in part explained by double reduction events although other mechanisms could also lead to segregation distortions, as in the case for loci under genetic selection.

To observe a double reduction, the formation of tetravalents is a prerequisite, and crossing overs have to occur between the locus and the centromere. Coefficient of double reduction at any locus depends on its genetics distance to the centromere. It's therefore expected lower for locus close to the centromere than for distal locus. Here, the three loci with the maximum value of α (1/6) could be located in the distal chromosomic regions. A genetic map based on SSR markers is being prepared on the studied segregating population and will make it possible to confirm these hypotheses.

Spontaneous tetraploids are formed though a variety of pathways (Harlan and De Wet 1975; Ramsey and Schemske 1998), one of which involves the union of two unreduced gametes in a single step. Because this mechanism would occur with the joint probability of two unlikely events, it is considered quite rare in natural populations. Alternatively, tetraploids may be produced in two steps via a triploid intermediary, through a process known as the triploid bridge (Husband 2004). The observation that some diploid female and male *D. alata* varieties in the CIRAD collection are able to produce unreduced gametes (Nemorin et al., in prep) could lead to the assumption that this phenomenon is at the origin of polyploidy in *D. alata*.

Autopolyploidy confers several agronomic advantages such as increased cell size and changes in physiology and ecological tolerance. This could account for their better performances, high and stable tuber yield and tolerance to abiotic and biotic stresses. (Gallais 2003; Comai 2005; Paterson 2005; Stupar et al. 2007; Parisod et al. 2010; Arnau et al.2010). The three ploidy levels coexist at the *D. alata* diversification centre (Malapa et al. 2005), and the polyploid genotypes could be selected for their agronomic performances.

The cultivated forms of the *D.trifida* species have a regular ploidy level of 2n=4*x*= 80 (Bousalem et al. 2006). However, a recent study has demonstrated that wild diploid and triploid forms exist in French Guyana (Bousalem et al. 2010). In *D.trifida* the tetraploid forms could have been selected, as in the case of potato, because they provide tubers with better size and weight compared to diploid varieties (Ortiz 1998; Bradshaw and Ramsay 2005; Vreugdenhil et al. 2007).

Conclusion and future prospects

Segregation patterns of tetraploid *D. alata* varieties ($2n = 4x = 80$) were determined by studying SSR inheritance markers and by calculating double reduction coefficients. Analyses of these two parameters provide strong evidence that tetraploid *D. alata* are autotetraploid and not allotetraploid.

These results lead to new insights into the improvement of the yam, *D. alata*. Because heritability of characters depends of genetic variance, and greater variances are expected in autopolyploids, the characters should be more heritable. Moreover the genetic advance should be increased in presence of epistasis compared to allopolyploids.

In *D. alata* several ways could be exploited to obtain heterozygote tetraploid progenies: by crossing two tetraploids or by using the ability of some diploid varieties to produce unreduced gametes. Studies are in progress to understand the origin of 2n gametes (FDR or SDR) which allow to know the heterozygosity rate transmitted to progeny.

References

Abraham K, Nair PG (1991) Polyploidy and sterility in relation to sex in Dioscorea alata L. (Dioscoreaceae). Genetica 83:93-97

Andris M, Aradottir GI, Arnau G et al (2010) Permanent Genetic Resources added to Molecular Ecology Resources Database 1 June 2010 – 31 July 2010. Mol Ecol Resour 10:1106-1108

Arnau G, Nemorin A, Maledon E, Abraham K (2009) Revision of ploidy status of Dioscorea alata (Dioscoreaceae) by cytogenetic and microsatellite segregation analysis. Theor Appl Genet 118: 1239-1249

Arnau G, Abraham K, Sheela MN, Chair H, Sartie A, Asiedu R (2010) Yams. In:Root and Tuber Crops Handbook of Plant Breeding, pp 127-148

Arnau G, Lallemand J, Bourgoin M (2002) Fast and reliable strawberry cultivar identification using inter simple sequence repeat (ISSR) amplification. Euphytica 129: 69-79

Babaei A, Tabaei-Aghdaei SR, Khosh-Khui M, Omidbaigi R, Naghavi MR, Esselink GD, Smulders MJM (2007) Microsatellite analysis of Damask rose (Rosa damascena Mill.) accessions from various regions in Iran reveals multiple genotypes. BMC Plant Biol 7:12

Bradshaw JE, Ramsay G (2005) Utilisation of the Commonwealth Potato Collection in potato breeding. Euphytica 146: 9-19

Bretagnolle F, Thompson JD (1995) Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. New Phytol 129: 1-22

Bousalem M, Arnau G, Hochu I, Arnolin R, Viader V, Santoni S, David J (2006) Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam Dioscorea trifida and a new basic chromosome number in the Dioscoreae. Theor Appl Genet 113:439-451

Bousalem M, Viader V, Mariac C, Gomez RM, Hochu I, Santoni S, David J (2010) Evidence of diploidy in the wild Amerindian yam, a putative progenitor of the endangered species Dioscorea trifida (Dioscoreaceae). Genome 53: 371-383

Catalán P, Segarra-Moragues JG, Palop-Esteban M, Moreno C, González-Candelas F (2006) A Bayesian Approach for Discriminating Among Alternative Inheritance Hypotheses in Plant Polyploids: The Allotetraploid Origin of Genus Borderea (Dioscoreaceae). Genetics172:1939-1953

Comai L (2005) The advantages and disadvantages of being polyploid. Nat Rev Genet 6: 836-846

Esselink GD, Nybom H, Vosman B (2004) Assignment of allelic configuration in polyploids using the MAC-PR (microsatellite DNA allele counting—peak ratios). Theor Appl Genet 109: 402-408

Estoup A, Tailliez C, Cornuet JM, Solignac M (1995) Size homoplasy and mutational processes of interrupted microsatellites in two bee species, Apis mellifera and Bombus terrestris (Apidae). Mol Biol Evol 12: 1074-1084

Ferrante SP, Lucretti S, Reale S, De Patrizio A, Abbate L, Tusa N, Scarano MT (2010) Assessment of the origin of new citrus tetraploid hybrids (2n 5 4x) by means of SSR markers and PCR based dosage effects. Euphytica 173:223-233

Fisher RA (1947) The theory of linkage in polysomic inheritance. Philos. Trans. R. Soc. Ser. B 233: 55-87

Gallais A (2003) Quantitative genetics and breeding methods in autopolyploid plants. Editions Quae, 516 p.

Grant V (1981) Plant speciation. 2nd ed. Columbia University Press, New York

Hackett CA, Luo ZW (2003) TetraploidMap: construction of a linkage map in autotetraploid species. J Hered. 94:358–359

Harlan JR, deWet JMJ (1975) On Ö. Winge and a Prayer: The origins of polyploidy. The Botanical Review 41: 361-390

Helsen P, Verdyck P, Tye A, Van Dongen S (2009) Low levels of genetic differentiation between Opuntia echios varieties on Santa Cruz (Galapagos). Plant Syst Evol 279:1–10

Husband BC (2004) The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biol J Linnean Soc 82: 537–546

Jackson RC, Jackson JW (1996) Gene segregation in autotetraploids: prediction from meiotic configurations. Am J Bot 83: 673–678

Jannoo N, Grivet L, David J, D'Hont A, Glaszmann JC (2004) Differential chromosome pairing affinities at meiosis in polyploid sugarcane revealed by molecular markers. Heredity 93: 460–467

Kass RE, Raftery AE (1995) Bayes Factors. J Am Stat Assoc 90: 773-795

Landergott U, Naciri Y, Schneller JJ, Holderegger R (2006) Allelic configuration and polysomic inheritance of highly variable microsatellites in tetraploid gynodioecious Thymus praecox agg. Theor Appl Genet 113: 453–465

Lebot V (2009) Tropical root and tuber crops: cassava, sweet potato, yams and aroids. Wallingford (ed) CABI, XIX, 413 pp.

Lumaret R, Borrill M (1988) Cytology, genetics, and evolution in the genus Dactylis. Crit Rev Plant Sci 7: 55–91

Luo ZW, Hackett CA, Bradshaw JE, McNicol JW, Milbourne D (2000) Predicting parental genotypes and gene segregation for tetrasomic inheritance. Theor Appl Genet 100: 1067–1073

Luo ZW, Hackett CA, Bradshaw JE, McNicol JW, Milbourne D (2001) Construction of a Genetic Linkage Map in Tetraploid Species Using Molecular Markers. Genetics 157: 1369-1385

Luo ZW et al. (2006) Constructing genetic linkage maps under a tetrasomic model. Genetics 172:2635–2645

Malapa R, Arnau G, Noyer JL, Lebot V (2005) Genetic diversity of the greater yam (Dioscorea alata L.) and relatedness to D. nummularia Lam. and D. transversa Br. as revealed with AFLP markers. Genet Resour Crop Evol 52:919–929

Mather K (1936) Segregation and linkage in autotetraploids. J. Genet. 32: 287–314

Misuki I, Tani N, Ishida K, Tsumura Y (2005) Development and characterization of microsatellite markers in a clonal plant, Dioscorea japonica Thunb. Mol Ecol Notes 5:721–723

Olson MS (1997) Bayesian procedures for discriminating among hypotheses with discrete distributions: inheritance in the tetraploid Astilbe biternub. Genetics 147:1933–1942

Ortiz R (2010) Potato Breeding via Ploidy Manipulations. Plant Breeding Reviews, Volume 16 (ed J. Janick)

Parisod C, Holderegger R, Brochmann C (2010) Evolutionary consequences of autopolyploidy. New Phytol 186: 5–17

Paterson AH (2005) Polyploidy, evolutionary opportunity, and crop adaptation. Genetica 123: 191–196

Ramsey J, Schemske DW (1998) Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants. Annu Rev Ecol Syst 29: 467-501

Rieseberg LH, Doyle JJ (1989) Tetrasomic segregation in the naturally occurring autotetraploid Allium nevii alliance. Hereditas 111: 31–36.

Ronfort JL, Jenczewski E, Bataillon T, Rousset F (1998) Analysis of population structure in autotetraploid species. Genetics 150: 921–930

Scarcelli N, Daı¨nou O, Agbangla C, Tostain S, Pham JL (2005) Segregation patterns of isozyme loci and microsatellite markers show the diploidy of African yam Dioscorea rotundata (2n = 40). Theor Appl Genet 111:226–232

Segraves KA, Thompson JN, Soltis PS, Soltis DE (1999) Multiple origins of polyploidy and the geographic structure of Heuchera grossulariifolia. Mol Ecol 8: 253–262.

Soltis DE, Riesberg LH (1986) Autopolyploidy in Tolmiea mziesii (Saxifragaceae): genetic insights from enzyme electrophoresis. Am J Bot 73:310–318

Soltis DE, Soltis PS (1989) Genetic consequences of autopolyploidy in Tolmiea (Saxifragaceae). Evolution 43: 586–594

Stebbins GL (1950) Variation and evolution in plants. Columbia University Press, New York, 643 pp

Stift M, Berenos C, Kuperus P, van Tienderen PH (2008) Segregation models for disomic, tetrasomic and intermediate inheritance in tetraploids: a general procedure applied to Rorippa (yellow cress) microsatellite data. Genetics 179: 2113–2123

Stupar RM, Bhaskar PB, Yandell BS, Rensink WA, Hart AL, Ouyang S, Veilleux RE, Busse JS, Erhardt RJ, Buell CR, Jiang J (2007) Phenotypic and Transcriptomic Changes Associated With Potato Autopolyploidization. Genetics176:2055-2067

Tostain S, Scarcelli N, Brottier P, Marchand JL, Pham JL, Noyer JL (2006) Development of DNA microsatellite markers in tropical yam (Dioscorea sp.). Mol Ecol Notes 6:173–175

Vreugdenhil D, Bradshaw J, Gebhardt C, Govers F, MacKerron DKL, Taylor MA, Ross HA (2007) Potato biology and biotechnology. Advances and perspectives. Elsevier, Oxford ISBN:13-978-0-444-51018-1

Watson LE, Elisens WJ, Estes JR (1991) Electrophoretic and cytogenetic evidence for allopolyploid origin of Manhullia mohrii (Asteraceae). Am J Bot 78:408–416

Wolf PG, Soltis DE, Soltis PS (1990) Chloroplast DNA and allozymic variation in diploid and autotetraploid Huechera grossulariifolia (Saxifragaceae). Am J Bot 77: 232–244

Wu RL, Gallo-Meagher M, Littell RC, Zeng Z-B (2001) A general polyploid model for analyzing gene segregation in outcrossing tetraploid species. Genetics 159: 869–882

Wu SS, Wu RL, Ma CX, Zeng Z-B, Yang M et al. (2001) Amultivalent pairing model of linkage analysis in autotetraploids. Genetics 159: 1339–1350.

Tables

Table 1 Distribution of expected and observed genotypes in the progeny "Tepuna X Noulelcae" at the mDaCIR108 locus under tetrasomic and disomic inheritance. Likelihoods for all the possible segregation patterns are given and the Bayes factor is computed.
Bayesian probabilities (36 values) for disomic inheritance are given for the parental set bcdd X abd0, but probabilities for the 16 other possible parental combinations have been taking into account; given a Bayesian probability total for disomic inheritance including 576 probabilities (36 X 16).

Parental genotype	Progeny genotype													Likelihood
	d	cd	bd	bc	bcd	ad	acd	abd	abc	abcd	ab	b	ac	$P = 0.01$
Observed genotypes (N=169)														
	9	6	22	4	27	11	23	38	8	17	4			
probabilities of expected progeny under tetrasomic inheritance (4*x*, given in 1/36)														
bcdd x abd0	1	2	8	1	6	2	4	7	2	3				10^{-19}
bbcd x aabd			3		3		3	12	6	6	3			10^{-45}
bbcd x abbd			8	2	7		1	7	4	4	2	1		10^{-43}
bbcd x abdd		1	9		8		2	8	2	5	1			10^{-32}
bbcd x abd0		1	8	2	6		2	7	4	3	2	1		10^{-32}
bccd x abd0		3	3	3	9		5	3	5	4			1	10^{-42}
bccd x abdd		3	3		12		6	3	3	6				10^{-43}
bccd x abbd			3	3	12		3	3	6	6				10^{-51}
bccd x aabd			1		5		8	5	8	8			1	10^{-59}
bcdd x aabd			3		3	3	6	12	3	6				10^{-30}
bcdd x abbd			9	1	8	1	2	8	2	5				10^{-28}
bcdd x abdd	1	2	8		7	2	4	7	1	4				10^{-23}
bcd0 x abd0	1	2	7	2	5	2	3	5	3	2	2	1	1	10^{-26}
bcd0 x abdd	1	2	8		7	2	4	6	2	3	1			10^{-20}
bcd0 x abbd			8	2	7	1	2	6	4	3	2	1		10^{-29}
bcd0 x aabd			3		3	3	5	9	5	4	3		1	10^{-30}
Distribution of expected progeny under disomic inheritance (4*x*, 1/16) for parental set bcdd x abd0														
bc/dd x a0/bd		2			2	4	2	4		2				10^{-45}
bc/dd x ab/0d			4		4		4	4						10^{-76}
bc/dd x ad/0b		2	4		2		2	4		2				10^{-45}
bc/dd x 0b/ad		2	4		2		2	4		2				10^{-45}
bc/dd x 0d/ab			4		4		4	4						10^{-76}
bc/dd x bd/a0		2	4		2		2	4		2				10^{-45}
bd/cd x a0/bd	1	1	3	1	2	1	1	3	1	2				10^{-25}
bd/cd x ab/0d			4	1	3	2	2	2	1	1				10^{-28}
bd/cd x ad/0b	1	1	3		3	1	1	3	2	1				10^{-29}
bd/cd x 0b/ad	1	1	3		3	1	1	3	2	1				10^{-29}
bd/cd x 0d/ab			4	1	3	2	2	2	1	1				10^{-28}
bd/cd x bd/a0	1	1	3	1	2	1	1	3	1	2				10^{-25}
bd/cd x a0/bd	1	1	3	1	2	1	1	3	1	2				10^{-25}
bd/cd x ab/0d			4	1	3	2	2	2	1	1				10^{-28}
bd/cd x ad/0b	1	1	3		3	1	1	3	2	1				10^{-29}
bd/cd x 0b/ad	1	1	3		3	1	1	3	2	1				10^{-29}
bd/cd x 0d/ab			4	1	3	2	2	2	1	1				10^{-28}
bd/cd x bd/a0	1	1	3	1	2	1	1	3	1	2				10^{-25}
cd/bd x a0/bd	1	1	3	1	2	1	1	3	1	2				10^{-25}
cd/bd x ab/0d			4	1	3	2	2	2	1	1				10^{-28}
cd/bd x ad/0b	1	1	3		3	1	1	3	2	1				10^{-29}
cd/bd x 0b/ad	1	1	3		3	1	1	3	2	1				10^{-29}
cd/bd x od/ab			4	1	3	2	2	2	1	1				10^{-28}
cd/bd x bd/a0	1	1	3	1	2	1	1	3	1	2				10^{-25}
cd/bd x a0/bd	1	1	3	1	2	1	1	3	1	2				10^{-25}

cd/bd x ab/0d			4	1	3	2	2	2	1	1	10^{-28}
cd/bd x ad/0b	1	1	3		3	1	1	3	2	1	10^{-29}
cd/bd x 0b/ad	1	1	3		3	1	1	3	2	1	10^{-29}
cd/bd x 0d/ab			4	1	3	2	2	2	1	1	10^{-28}
cd/bd x bd/a0	1	1	3	1	2	1	1	3	1	2	10^{-25}
dd/bc x a0/bd		2	4		2		2	4		2	10^{-45}
dd/bc x ab/0d			4		4		4	4			10^{-76}
dd/bc x ad/0b		2	4		2		2	4		2	10^{-45}
dd/bc x 0b/ad		2	4		2		2	4		2	10^{-45}
dd/bc x 0d/ab			4		4		4	4			10^{-76}
dd/bc x bd/a0		2	4		2		2	4		2	10^{-45}
Bayes factor: analysis integrating all the possibilities											**4,60E+05**

Table 2 Bayes factors testing the likelihood of disomic versus polysomic 4x inheritance patterns in a segregating tetraploid population of *Dioscorea alata* on 20 microsatellite loci.

Nb A.: allele number; Σauto: sum of all Bayesian probabilities under autotetrasomic inheritance; Σallo homoplasy: sum of all Bayesian probabilities under disomic inheritance considering homoplasy; Σallo no homoplasy: sum of all Bayesian probabilities under disomic inheritance considering no homoplasy hypothesis; BF: Bayes factor under homoplasy or no homoplasy hypothesis,* BF>200 strong evidence.

Locus	Nb A.	Σauto	Σallo homoplasy	Σallo no homoplasy	BF homoplasy	BF no homoplasy
DIJ0443	5	3.37E-33	7.98E-76	7.98E-76	4.22E+42*	4.22E+42*
DIJ0342	5	3.06E-26	7.66E-64	7.66E-64	3.99E+37*	3.99E+37*
Dpr3B12	5	3.79E-17	5.65E-35	2.26E-73	6.71E+17*	1.68E+56*
Dab2D11	4	5.57E-11	2.38E-25	2.91E-33	2.34E+14*	1.91E+22*
mDaCIR8	4	3.54E-16	8.62E-26	1.00E-48	4.11E+09*	3.54E+32*
mDaCIR108	4	2.06E-19	4.48E-25	2.41E-45	4.60E+05*	8.55E+25*
mDaCIR51	4	1.37E-21	3.63E-27	5.39E-80	3.77E+05*	2.54E+58*
Dab2D07	4	3.73E-20	2.26E-24	9.30E-53	1.65E+04*	4.01E+32*
mDaCIR179	4	7.28E-13	4.47E-16	3.71E-79	1.63E+03*	1.96E+66*
mDaCIR66	4	1.26E-08	4.36E-11	4.36E-11	2.89E+02*	2.89E+02*
Dpr3F04	4	1.81E-06	1.14E-08	1.36E-82	1.59E+02	1.33E+76*
Da2F10	4	9.60E-18	5.15E-17	1.75E-111	1.86E-01	5.49E+93*
DIJ0012	3	1.30E-06	3.69E-08	1.64E-18	3.52E+01	7.93E+11*
Da3G04	3	2.93E-05	1.08E-06	1.38E-17	2.73E+01	2.12E+12*
Da1C12	3	1.42E-05	1.70E-06	1.82E-09	8.35E+00	7.80E+03*
Dab2E07	3	1.79E-06	3.75E-07	3.51E-12	4.77E+00	5.10E+05*
Dab2D08	3	4.90E-04	5.10E-04	1.33E-12	9.61E-01	3.68E+08*
mDrCIR81	3	9.00E-09	9.75E-09	2.21E-36	9.23E-01	4.07E+27*
Da1F08	3	7.56E-06	1.79E-05	2.13E-15	4.22E-01	3.55E+09*
Dpr3E10	3	2.19E-10	6.98E-09	6.04E-48	3.14E-02*	3.63E+37*

Table 3 Double reduction coefficient
Nb A.: allele number; gen.♂ and gen.♀: parental genotypes and its goodness of fit "sign."; α: double reduction coefficient and its significance, Nb DR: number of genotypes that can only be obtained by double reduction

Locus	Nb A.	gen.♂	gen.♀	sign.	α	sign. α	Nb DR
DIJ0443	5	abd0	ccce	> 0.9995	0.05	1.179 E-7*	5
DIJ0342	5	abd0	ccce	> 0.9995	0.04	1.692 E-9*	4
Dpr3B12	5	bcde	acee	> 0.9995	0.04	5.526 E-5*	9
Dab2D11	4	abcd	bbd0	0.58	0.03	0.0497*	2
mDaCIR8	4	bcc0	abdd	>0.9995	0.16	0.0446*	27
mDaCIR108	4	bcd0	abdd	0.998	0.05	1.544 E-8*	4
mDaCIR51	4	acdd	bcc0	>0.9995	0.09	0*	12
Dab2D07	4	bcd0	ad00	0.52	0.02	0.049*	1
mDaCIR179	4	abbc	abdd	0.97	0.1	0*	7
mDaCIR66	4	bbcd	abbd	0.19	0	1.0	0
Dpr3F04	4	aaac	bbdd	0.28	0.06	1.152 E-12*	2
Da2F10	4	accd	aabd	> 0.9995	0.16	0*	11
DIJ0012	3	abbc	bcc0	> 0.9995	0.11	0*	5
Da3G04	3	abbc	ccc0	0.55	0.14	4.649E-12*	11
Da1C12	3	aabc	bbc0	>0.9995	0.04	4.688E-6*	3
Dab2E07	3	aabb	abcc	0.93	0.10	0.1900	0
Dab2D08	3	aabc	aaac	0.19	0.08	0.466	0
mDrCIR81	3	abb0	aac0	> 0.9995	0.12	9.224 E-10*	2
Da1F08	3	bbcc	abc0	0.42	0.13	0.1803	0
Dpr3E10	3	aabb	aacc	>0.9995	0.16	0.038*	0

* significant at P≤0.05

1.2. Etude cytogénétique de la méiose des variétés tétraploïdes de D. alata

Cette partie est présentée sous forme d'article : « Meiosis and sexual fertility of autotetraploid clones of greater yam *Dioscorea alata* L.» accepté dans *Genetic Ressources and Crop Evolution.*

1.2.1. Résumé

Les méioses des mâles tétraploïdes (2n = 80) de *D. alata* ont été étudiées pour la première fois. Durant la métaphase I, les chromosomes étaient appariés le plus souvent sous forme de 6-8 quadrivalents, les autres appariements étaient des bivalents, aucun trivalent ni univalents n'ont été observés. L'anaphase I et les autres étapes de la méiose étaient normales. L'observation de quadrivalents chez les tétraploïdes fournit la preuve cytologique de l'autotétraploïdie de *D. alata*. Les mâles et femelles autotétraploïdes sont très fertiles et produisent des graines viables par pollinisation artificielle. Des croisements entre diploïdes (2n = 40) et tétraploïdes (2n = 80) ont été réalisés avec succès via le sauvetage d'embryons immatures, produisant des descendances triploïdes (2n = 60). La découverte de la fertilité des autotétraploïdes pourrait permettre d'initier l'amélioration au niveau polyploïde chez *D. alata* par hybridation artificielle. Le faible nombre de quadrivalents et la fertilité élevée des autotétraploïdes serait le résultat d'une diploïdisation partielle des méioses. Ces connaissances réfutent également la probable origine allopolyploïde de *D. alata* à partir de deux géniteurs potentiels.

Mots clés: Autotétraploïdie; *Dioscorea alata;* grande igname; amélioration polyploïde; quadrivalents

1.2.2. Article II

Meiosis and sexual fertility of autotetraploid clones of greater yam *Dioscorea alata* L.

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Genetic Ressources and Crop Evolution (2012)

accepté le 18/2/ 2012

Abstract

Meiosis in the tetraploid (2n = 80) males of *Dioscorea alata* L. was investigated for the first time. During metaphase I, the chromosomes were associated mostly in 6 – 8 quadrivalents and the remaining ones as bivalents with no trivalents or univalents. Anaphase I and subsequent stages of meiosis were normal. The observation of quadrivalents in the tetraploids provides cytological evidence for autotetraploidy. The autotetraploid males and females were highlyfertile and they produced viable seeds on artificial pollination. Pollination between diploids (2n=40) and tetraploids (2n = 80) were also successful via embryo rescue, producing triploid (2n= 60) progenies. The discovery of fertile autotetraploids could initiate polyploidy breeding in *D. alata* by conventional hybridization for the first time. The reduced number of quadrivalents and the high fertility of the autotetraploids are thought to be the result of partial diploidization of meiosis. The findings also refute the assumption of the allopolyploid origin of *D. alata* from two putative progenitors.

Key words: Autotetraploidy; *Dioscorea alata;* Greater yam; Polyploidy breeding; Quadrivalents

Introduction

The genus Dioscorea (family Dioscoreaceae), consisting of the group of yam species, is pantropical in distribution with over 650 species (Degrass 1993). Yams are dioecious species and the plants are characterized by weak aerial stems that twine on supports, producing underground tubers or rhizomes. Dioscorea alata L. or greater yam is second in importance among the major food yams cultivated in the tropics for its starchy tubers. It belongs to the Asiatic group of yams of the Old World, which is considered to have originated in the Indo-Malayan centre (Coursey 1979). Different ploidy levels were reported within several Asiatic, African and American Dioscorea species (Martin and Ortiz 1963 and references therein). In *D. alata* the earliest reports of chromosome numbers during 1930s varied as 2n = 20, 30, 40, 50, 60 and 70 (Martin and Ortiz 1963) but recent studies during 1990s on large numbers of accessions in India (Abraham and Nair 1991), Pacific (Malappa et al. 2005), Africa (Egesi et al. 2002; Obidiegwu et al. 2010) and Caribbean (Gamiette et al. 1999; Arnau et al. 2009) have consistently reported only threechromosome numbers, 2n = 40, 60 and 80. Although D. alata has been cultivated throughout the tropics as a clonal crop, genetic improvement by hybridization was never practiced as there was no natural seed set observed anywhere, leaving the impression that the species was sterile (Martin 1977). However, investigations at the International Institute of Tropical Agriculture (IITA) Nigeria and Central Tuber Crops Research Institute (CTCRI), India had recorded trace fertility in a few females withnominal fruit set and seed production during the 1970s (CTCRI 1974; IITA 1977). The first reports of successful hand pollination between 2n = 40 males and females, resulting in the production of large seedling populations were from CTCRI, India (Abraham et al. 1986; Bai and Jos 1986). Cytological screening of the flowering collections revealed that the distribution of ploidy levels was not similar in the two sexes of this dioecious species. While among females higher ploids (2n = 60 and 80) were common, 2n = 60 types were rare and 2n = 80 types were not even observed among the males (Abraham and Nair 1991). Recently, studies were conducted in the Pacific germplasm accessions of D. alata for the first time, under the Indo-French collaborative research project between Central Tuber Crops Research Institute (CTCRI), India and the Centre de Coopération Internationale en Recherche Agronomique pour le Developpement (CIRAD), France at the CIRAD station, Guadeloupe,French West Indies. During the study, the microsatellite segregation analysis of the sexual progenies of 2n = 40 parents revealed the basic chromosome number of D. alata as x = 20 (Arnau et al. 2009), revising the age old consideration of x = 10 (Coursey 1979). Like the Indian genetic stocks, the Pacific genetic stocks also consisted of only three ploidy levels (2n = 40, 60 and 80) but

flowering males and females were normally available in all the ploidy levels. Hence the meiosis of the 2n = 80 male was investigated and pollination studies were conducted for the first time, using 2n = 80 males and females. The studies on the Pacific germplasm of *D. alata* provided new evidence on the level and nature of ploidy in the species. This paper mainly reports the meiosis and the sexual fertility studies in the 2n = 80 clones of D. alata, providing cytological evidence for its autopolyploid nature and thereby making a correction of the conclusions by the senior author in two of his earlier publications (Abraham and Nair 1991; Abraham 1998).

Materials and methods

The Pacific germplasm accessions of D. alata maintained at the Station De Roujol, Centre de Cooperation International en Recherche Agronomique pour le Developpement (CIRAD), Guadeloupe, French West Indies were utilized for the study. Cytological analyses were performed on the tetraploid clones. For studying meiosis, male flower buds were fixed in the morning (between 9 – 10 am) in alcohol-acetic acid (3:1), adding a tinge of ferric acetate. Smears were prepared after 24 hr in 2 per cent acetocarmine using the method described by Abraham and Nair (1991). Chromosome associations were recorded from 50 PMCs (pollen mother cells) of metaphase I stage. Pollen fertility was estimated by stainability in acetocarmine, counting at least 250 pollen grains from random microscopic fields. Artificial pollinations were done by the 'pencil method' described by Abraham and Nair (1990). Fruit set was recorded 3 – 4 weeks after pollination and seed set was recorded when the capsules were harvested and dried. Seed germination was tested in a sand-soil mixture inside a glass house.

Results

Meiosis of 2n=80 males The metaphase I in a large number of PMCs of the 2n = 80 males was examined. In all the PMCs, chromosomes were associated as bivalents and quadrivalents. The number of quadrivalents in the PMCs ranged from 6 –10 but the most frequent numbers of quadrivalents were 6 – 8 (Figs A and B; Table 1). Apart from quadrivalents, all the remaining chromosomes in the PMCs paired as bivalents. No univalent was observed, except for some loosely associated bivalents giving the appearance of univalents. Also no trivalent was observed in any of the PMCs. Occasionally two quadrivalents would remain adjacently, giving the appearance of a higher association. Although quadrivalents were observed invariably during metaphase I, they segregated normally during anaphase I and the subsequent

stages were also normal, resulting in well formed pollen grains. Fertility of 2n = 80 males and females The pollen grains of 2n = 80 males were well-filled and highly stainable (80-90 %) as in the case of those of 2n = 40 males. Pollen fertility was also tested by conducting pollination studies between 2n = 80 males and females. The pollen grains of 2n = 80 male was successful in effecting fruit set (56-70 %) and producing viable seeds (70-80 %) in 2n = 80 females. In pollinations on 2n = 40 females with 2n = 80 males, the fruit set was 30-40 per cent and seed set was 15-20 per cent. The reciprocal crosses (2n = 80 females with 2n = 40 males) were not successful. The pollen grains of 2n = 60 males were apparently sterile as they were non-stainable and devoid of cytoplasm. They were not successful in pollinating any females of any ploidy level. So also, the 2n = 60 females never formed any viable seed when pollinated with fertile males of 2n = 40 or 80, although fruits were formed in some cases. Obviously they are sterile due to the trivalent formation and meiotic irregularities (Abraham 1998). As a result of the successful pollinations between 2n = 80 parents, progeny with 2n = 80 constitution could be produced for the first time. So also successful pollinations between 2n = 40 females and 2n = 80 males produced 2n = 60 progeny by using embryo rescue. These studies have opened up the possibility of polyploidy breeding in D. alata by conventional hybridization.

Discussion

The basic chromosome number of the Old World Dioscorea species was considered for long as x = 10 (Coursey1979; Essad 1984). Recently, segregation studies of microsatellite markers in the sexual progeny have changed this concept in the case of several yam species. In the Asian greater yam D. alata, the so called tetraploid forms (2n = 40) were proved to be diploids and the basic chromosome number was revised as x = 20 (Arnau et al. 2009). So also, in the African white yam, Dioscorea rotundata Poir.(Scarcelli et al. 2005) and the American yam, Dioscorea trifida L. (Bousalem et al. 2006) the basic chromosome numbers were revised as x = 20, based on marker segregation evidence. The present study is the first report of the meiosis of the tetraploid (2n = 80) males of D. alata which were discovered among the Pacific germplasm accessions. The consistent observation of 6 – 8 quadrivalents in the PMCs is the first cytological evidence for the autotetraploid nature of the 2n = 80 males, which were found to be highly fertile. The high sexual fertility of the autotetraploid D. alata may be ascribed to the absence of univalents and/or trivalents. Studies among autotetraploid plant species have shown that the cause of low sexual fertility is the formation of univalents and trivalents rather than quadrivalents, as the former would cause unbalanced segregation of chromosomes in the sister cells whereas quadrivalent mis-disjunction was not a significant factor (Narasinga Rao

and Pantulu 1982). The low frequency of quadrivalents, absence of univalents and trivalents and the high sexual fertility observed in the autotetraploids D. alata indicate that, over the years of cultivation, a partial diploidisation of meiosis has taken place among the vegetatively propagated cultivars. In the induced autotetraploids of perennial rye, substantial stability in meiosis towards diploidization was observed progressively within three generations, by the significant reduction in univalents and other meiotic aberrations as well as improvement of seed set (Akgun and Tosun 2007). Similar diploidization of meiosis was observed in Arabidopsis thaliana, in which the multivalents were very few at metaphase I of the established autotetraploids, compared to the newly generated autotetraploids in which a very high level of multivalent association was observed (Santos et al. 2003). Studies among induced autopolyploids indicate that meiotic diploidization occurs relatively rapidly, with or without accompanying mutagenesis to induce structural chromosome rearrangements (Gillies 1989). Diploidization of autopolyploids is considered to have occurred quite frequently during the evolution of plant species (Levin 2002) and most likely, the mechanism has played a significant role in the formation of sexually fertile autotetraploids in D. alata. As a continuation of the present study, tetraploids of D. alata have been artificially induced by colchicine treatment and it would be interesting to study their meiotic behavior to observe the extent of quadrivalent formation in the newly formed autotetraploids. Early taxonomic considerations have attributed a hybrid origin of D. alata from two wild species such as Dioscorea persimilis Prain and Burk. and Dioscorea hamiltonii. Hook. Although with no evidence, the above hypothesis was widely accepted until the recent evidence (Arnau et al. 2009), influencing the cyto-taxonomical studies in the species. The new evidence for x = 20 in D. alata disproves the earlier conclusions on the diploid like meiotic behavior of the 2n = 40 males of D. alata, attributed to possible allotetraploidy (Abraham and Nair 1991) and the trivalent formation of 2n = 60 male indicated as auto-allohexaploidy (Abraham 1998). The AFLP fingerprinting studies among the Pacific varieties of D. alata have also shown that the species is genetically distant to D. persimilis but close to Dioscorea nummularia Lam. and Dioscorea transversa Br. (Malapa et al. 2005), further disproving the hypothesis of the allopolyploid ancestry of the species with D. persimils as one of the progenitors. Greater genetic variation was observed for D. alata cultivars in the Pacific, and it was proposed that Melanesia is the area of cultivar diversification of D. alata (Martin and Rhodes 1977) and hence, a third centre of diversity for the species was suggested in the Pacific region especially around Papua New Guinea. According to Coursey (1979), D. alata must have been taken across the Pacific by about 3500 BP and associated with the Melanesian culture even earlier.

Among the spatially isolated Asian and Pacific genetic stocks, much genetic diversification has been detected (Abraham and Arnau 2009). But it was among the Pacific accessions that the flowering types of both sexes in all ploidy levels were preserved. Nevertheless seed set was unknown to Pacific islanders as only the clones were cultivated. The joint study of CTCRI and CIRAD has discovered the unique genetic stocks of the Pacific gene pool for cytological, breeding and genetic divergence investigations (Abraham and Arnau 2009). Although 2n = 80 types were observed among the Indian accessions, their non-flowering nature has not revealed their sexes which had led to the confusion about the differential distribution of polyploidy among the sexes of the Indian accessions in the earlier studies (Abraham and Nair 1991). The discovery of sexually fertile natural autotetraploids of D. alata is of great importance in the genetic improvement of this crop in which polyploidy breeding by conventional hybridization could be initiated for the first time producing tetraploids and triploids which are more vigorous and higher yielding than diploids (Abraham and Arnau 2009; Arnau et al. 2010).

Acknowledgements: The work was conducted under the project no. 3000–B1 of the Indo-French Centre for the Promotion of Advanced Research (IFCPAR), New Delhi, India. The authors express their sincere gratitude to IFCPAR for the financial support of the project.

References

Abraham K. (1998) Occurrence of hexaploid males in Dioscorea alata L. Euphytica 99:5-7

Abraham K, Arnau G (2009) Use of DNA markers for genetically improving the productivity, palatability, storability and dry matter content of tubers of greater yam. Report of project no. 3000-B1. Indo- French Society for the Promotion of Advanced Research (IFCPAR). New Delhi, India. 82 pp

Abraham K, Nair PG (1990) Floral biology and artificial pollination in Dioscorea alata L. Euphytica 48:45-51

Abraham K, Nair PG (1991) Polyploidy and sterility in relation to sex in Dioscorea alata L. (Dioscoreaceae). Genetica 83:93-97

Abraham K, Nair SG, Sreekumari MT, Unnikrishnan M (1986) Seed set and seedling variation in greater yam (Dioscorea alata L.). Euphytica 35:337-343

Akgun I, Tosun M (2007) Seed set and some cytological characters in different generations of autotetraploid perennial rye (Secale montanum Guss) New Zealand J Agric Res 50:339- 346

Arnau G, Abraham K, Sheela MN, Hana Chair, Alieu Sartie, Robert Asiedu (2010) Yams. In: Bradshaw JE (ed) Root and tuber crops. Handbook of plant breeding Volume 7. Springer, pp 127-148

Arnau G, Nemorin A, Maledon E, Abraham K (2009) Revision of ploidy status of Dioscorea alata L. (Dioscoreaceae) by cytogenetic and microsatellite segregation analysis. Theor Appl Genet 118:1239-1249

Bai KV, Jos JS (1986) Female fertility and seed set in Dioscorea alata L. Trop. Agriculture, Trinidad 63(1):7-10

Bousalem M, Arnau G, Hochu I, Arnolin R, Viader V, Santonini S, David J (2006) Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam Dioscorea trifida and a new basic chromosome number in the Dioscoreae. Theor Appl Genet 113:439-451

Coursey DG (1979) Yams. In: Evolution of Crop Plants, (ed) NW Simmonds, Longman, London, pp 70-74, CTCRI (Central Tuber Crops ResearchInstitute), 1974. Annual Report. Trivandrum, India

Degrass L (1993) The Yam, a Tropical Root Crop. MacMillan, London 408 pp

Egesi CN, Pillay M, Asiedu R, Egunjobi JK (2002) Ploidy analysis in water yam, *Dioscorea alata* L. germplasm. Euphytica 128:225-230

Essad S (1984) Variation geographique des chromosomiques de base et polyploidie dans le genre Dioscorea a propos du denombrement des especes transversa Brown, pilosiuscula Bert. et trifida L. Agronomie 4(7):611-617

Gamiette F, Bakry F, Ano G (1999) Ploidy determination of some yam species (Dioscorea spp.) by flow cytometry and conventional chromosome counting. Genet Resour Crop Evol 46:19-27

Gillies CB (1989) Chromosome pairing and fertility in polyploids. In: Gillies CB (ed.) Fertility and chromosome pairing: Recent studies in plants and animals. CRC Press, Boca Raton, FL. pp 138 – 177, IITA (International Institute of Tropical Agriculture). 1977. Annual Report.

Ibadan, NigeriaLevin DA (2002) The evolution of polyploidy lineages. In: The role of chromosomal changes in plant evolution. Oxford university press, pp 163-184

Malapa R, Arnau G, Noyer JL, Lebot V (2005) Genetic diversity of the greater yam (Dioscorea alata L.) and relatedness to D. nummularia Lam. And D. transversa Br. as revealed with AFLP markers. Genet Resour Crop Evol 52:919-929

Martin FW, Ortiz S (1963) Chromosome numbers and behavior in some species of Dioscorea. Cytologia 28: 96-101

Martin FW, Rhodes AM (1977) Intra-specific classification of Dioscorea alata. Trop Agri (Trinidad) 54:1-13

Narasinga Rao PSRL, Pantulu JV (1982) Meiotic chromosome behaviour in autotetraploid pearl millet. Theor Appl Genet 62:345-351

Obidiegwu L, Rodriguez E, Ene-Obong E, Loureiro J, Muoneke C, Santos C, Kolesnikova-Allen M, Asiedu R (2010) Ploidy levels of Dioscorea alata L. germplasm determined by flow cytometry. Genet Resour Crop Evol 57:351-356

Santos JL, Alfaro D, Sanchez-Moran E, Armsrtong SJ, Franklin FCH, Jones GH (2003) Partial diploidization of meiosis in autotetraploid Arabidopsis thaliana.Genetics 165:1533-1540

Scarcelli N, Dainou O, Agbangla C, Tostain S, Pham JL (2005) Segregation patterns of isozyme loci and microsatellite markers show the diploidy of African yam Dioscorea rotundata (2n = 40). Theoretical and Applied Genetics 111: 226-232

Figures: PMCs of autotetraploid *Dioscorea alata* (2n = 80) showing quadrivalents and bivalents at Metaphase I (the bar indicates 10µ)

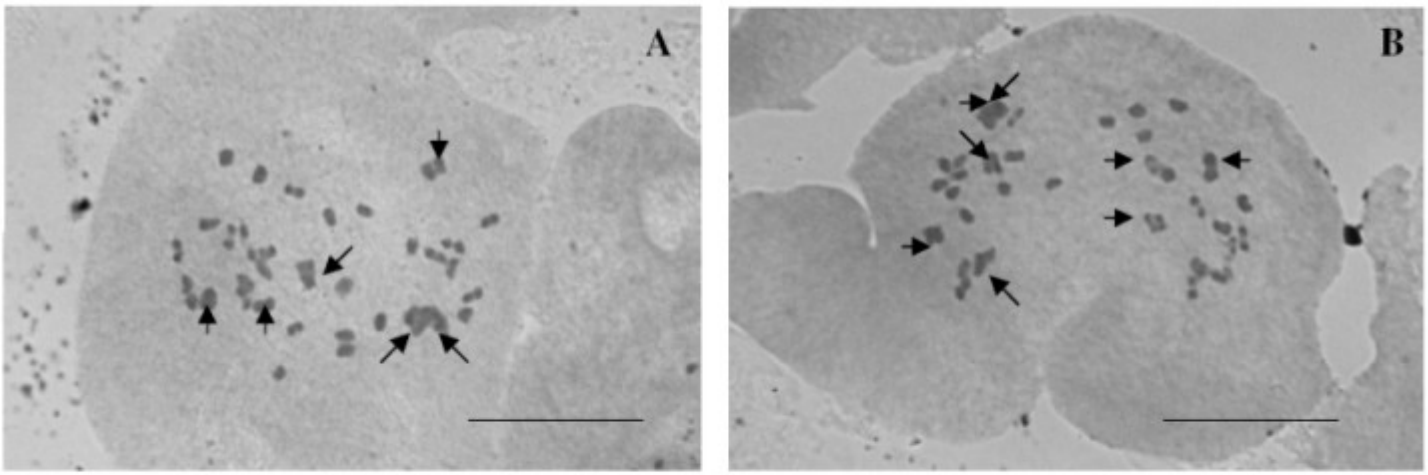

Fig A - a PMC showing 6 quadrivalents and 28 bivalents

Fig B - a PMC showing 8 quadrivalents and 24 bivalents

Table 1. Chromosome associations in 50 PMCs at metaphase I in the autotetraploid *D. alata*

Serial No	Chromosome association		Frequency of PMCs	
	IV	II	No	%
1	6	28	15	30.0
2	7	26	17	34.0
3	8	24	11	22.0
4	9	22	3	6.0
5	10	20	4	8.0
Mean	7.28	25.44		
Range	6 -10	20 -28		

PARTIE 2 : ORIGINE DES POLYPLOIDES DE *D. ALATA*

Cette partie est présentée sous forme d'un manuscrit intitulé « Microsatellite and flow cytometry analysis to understand the origin of *Dioscorea alata* polyploids » soumis à Annals of botany.

2.1. Résumé

Dioscorea alata est une espèce polyploïde avec des niveaux de ploïdie allant de diploïde (2*n* = 2*x* = 40) à tétraploïde (2*n* = 4*x* = 80). L'augmentation de la ploïdie est corrélée avec l'augmentation de la vigueur, des rendements plus élevés et plus stables et une augmentation de la tolérance aux stress biotiques et abiotiques. Des programmes d'amélioration au niveau polyploïde ont été initiés récemment avec la découverte de la fertilité des clones tétraploïdes. Le manque de connaissances sur l'origine des polyploïdes spontanés de *D. alata* (triploïdes et tétraploïdes) constitue une limite à l'efficacité de l'amélioration. L'objectif de cette étude est de comprendre la formation des polyploïdes de *D. alata* en utilisant la cytométrie en flux et les marqueurs microsatellites. Différentes descendances ont été générées par croisement - 2*x* x 2*x*, 2*x* x 4*x*, 3*x* x 2*x* - et analysées dans le but de comprendre les phénomènes d'incompatibilité au niveau de l'albumen et l'origine des gamètes via le taux d'hétérozygotie transmis à la descendance. Les résultats obtenus montrent que les polyploïdes de *D. alata* peuvent apparaître par polyploïdisation sexuelle *via* la formation de gamètes non réduits. Le pool triploïde se serait édifié et diversifié uniquement par formation de 2*n* gamètes chez des femelles diploïdes. Le pool tétraploïde pourrait ainsi être apparu par l'union de deux gamètes non réduits de parents diploïdes.

Mots clés: *Dioscorea alata*, incompatibilités d'albumen, 2*n* gamètes, polyploïdisation sexuelle bilatérale, origine des triploïdes

2.2. Manuscrit

Microsatellite and flow cytometry analysis to understand the origin of *Dioscorea alata* polyploids

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soumis le 8/10/2012

Abstract

Background and aims Dioscorea alata is a polyploid species with a ploidy level ranging from diploid ($2n = 2x = 40$) to tetraploid ($2n = 4x = 80$). Ploidy increase is correlated with better agronomic performances. The lack of knowledge about the origin of *Dioscorea alata* spontaneous polyploids (triploids and tetraploids) limits the efficiency of polyploid breeding. The objective of the present study was to use flow cytometry and microsatellite markers to understand the formation of *Dioscorea alata* polyploids.

Methods Different progenies generated by intracytotype crosses ($2x$ x $2x$) and intercytotype crosses ($2x$ x $4x$ and $3x$ x $2x$) were analysed in order to understand endosperm incompatibility phenomena and gamete origins via the heterozygosity rate transmitted to progeny.

Results This work shows that in a $2x$ x $2x$ cross, triploids with viable seeds are obtained only via a phenomenon of diploid female non-gametic reduction. The study of the transmission of heterozygoty made it possible to exclude polyspermy and polyembryony as the mechanisms at the origin of triploids. Non-obtention of seedlings by $3x$ x $2x$ cross made it possible to confirm the sterility of triploid females. Flow cytometry analyses carried out on the endosperm of seeds resulting from $2x$ x $4x$ crosses revealed endosperm incompatibility phenomena.

Conclusions The major conclusion is that the polyploids of *Dioscorea alata* would have appeared through the formation of unreduced gametes. The triploid pool would have been built and diversified through the formation of $2n$ gametes in diploid females as the result of the non-viability of seeds resulting from the formation of $2n$ sperm and of the non-viability of intercytotype crosses. The tetraploids would have appeared through bilateral sexual polyploidisation via the union of two unreduced gametes due to the sterility of triploids.

Keywords: *Dioscorea alata*, endosperm balance, $2n$ gametes, bilateral sexual polyploidisation, triploid origin

INTRODUCTION

Dioscorea alata is a monocot that belongs to the family Dioscoreaceae and to the gender Dioscorea. This gender includes more than 600 species (Ayensu and Coursey, l972) of which the three main cultivated ones are *Dioscorea rotundata, Dioscorea alata* and *Dioscorea trifida*. Yams are an important food crop in tropical and subtropical regions. They are dioecious herbaceous vines cultivated for their starchy tubers. They are exclusively propagated by vegetative multiplication by means of small tubers or small pieces of tubers. New combinations can be obtained via sexual reproduction, and breeding new cultivars has proven to be an efficient way of genetic improvement (Abraham and Nair, 1991; Egesi and Asiedu, 2002; Arnau et al., 2010, 2011).

D. alata is a polyploid species with diploid ($2n = 40$), triploid ($2n = 60$) and tetraploid ($2n = 80$) cytotypes (Arnau et al., 2009). Ploidy increase is correlated with growth vigour, higher and more stable tuber yield and increased tolerance to abiotic and biotic stress (Malapa et al., 2005; Lebot, 2009; Arnau et al., 2010). Recent studies have demonstrated a tetrasomic segregation for *D. alata* tetraploid clones ($2n = 4x = 80$) (Nemorin et al., 2012). Autotetraploidy confers several advantages of which hybrid vigour, also known as heterosis, is among the most common (Gallais, 2003; Comaï, 2005; Paterson, 2005; Stupar et al., 2007; Woodhouse et al., 2009; Parisod et al., 2010). Autotetraploids can maintain three or four different alleles at a given locus, implying that they will have higher heterozygosity than their diploid parents (Soltis et al., 2004). Because polyploid offspring have two or more copies of any particular gene, the offspring are protected from the deleterious effects of recessive mutations (Parisod et al., 2010).

Autotetraploids can be formed from diploids through a variety of ways (Harlan and de Wet, 1975; Ramsey and Schemske, 1998), one of which is thought to involve the combination of two unreduced ($2n$) gametes (bilateral sexual polyploidisation, BSP) (Bretagnolle and Thompson, 1995; Ramsey and Schemske, 1998). There are two major ways to produce $2n$ gametes: by first division restitution (FDR) or by second division restitution (SDR) (Mok and Peloquin, 1975). These two cytological events have different genetic consequences. In fact, FDR $2n$ gametes transmit the major part of parental heterozygosity to progeny, whereas SDR $2n$ gametes are rather homozygous. Because BSP would only occur with the joint probability of two lowly likely events, it is considered quite rare in natural populations (Husband, 2004). However, tetraploids have been obtained by BSP in several species such as *Dactylis*

glomerata (Bretagnolle and Lumaret, 1995), *Trifolium pratense* (Parrott and al., 1985) and diploid relatives of *Solanum tuberosum* (Mendiburu and Peloquin, 1977; Hutten et al., 1995).

Alternatively, tetraploids may be produced in two steps via a triploid intermediary, through a process known as the triploid bridge (unilateral sexual polyploidisation, USP). Tetraploids have been obtained by USP in several species such as *Cucumis sativus* (Diao et al., 2009), *Achillea borealis* (Ramsey, 2007) and *Chamerion angustifolium* (Husband, 2004). Triploids that are formed through the union of a haploid (x) and a diploid unreduced ($2x$) gamete are more or less fertile depending on the species (Ortiz and Vuylsteke, 1995; Burton and Husband, 2001; Park et al., 2002). Meiosis of triploids is irregular and results in a majority of aneuploid gametes. Euploid gametes that are haploid, diploid or triploid would be formed at the expected frequencies of $1/2^x$, $1/2^x + 1/2^x$, $1/2^x + 1/2^x + 1/2^x$, respectively (Lange and Wagenvoort, 1973), where x is the basic chromosome number. Depending on their fertility and the ploidy of their functional gametes (e.g., n=x, $2x$ or $3x$), triploids may produce tetraploids through the union of a balanced triploid gamete ($3x$) by a haploid gamete (x) of a diploid or a triploid. Non-gametic reduction in triploids can increase the frequency of balanced gametes ($2x$ and $3x$) by SDR and FDR, respectively, as is the case in *Chamerion angustifolium* (Husband, 2004).

Unions of gametes of different ploidy levels are expected to interfere with seed development. In angiosperms, seeds are obtained by double fertilisation (fertilisation of the oosphere, producing the embryo and fertilisation of the central cell, leading to the formation of the endosperm). Because the central cell of most flowering plant species is homodiploid ($2x$) and fertilised by a haploid spermatozoid (x), the resulting endosperm is triploid (2+1) and, therefore, genetically distinct from the diploid embryo (1+1). The endosperm of most angiosperms is triploid with a 2:1 ratio of the maternal to the paternal genome, although exceptions have been found in some species (Williams and Friedman, 2002; Mizuochi et al., 2009). Endosperm is particularly sensitive to ploidy misbalance (Khöler et al., 2010). An unbalanced endosperm may lead to an unviable seed (Costa et al. 2004). Deviations from the ratio of two maternal (2m) to one paternal (1p) genome in the endosperm can cause endosperm failure (Khöler et al., 2010). Increased contributions of maternal or paternal genomes inhibit proliferation of the endosperm or cause endosperm excess, respectively (Scoot et al., 1998).

D. alata breeding programmes were exclusively based on the creation of diploid varieties until 2006, although polyploidy has been acknowledged for a long time

(Ramachandran, 1968; Abraham and Nair, 1991). The first polyploid hybrids were recently created by conventional hybridisation, thanks to the discovery of the fertility of *D. alata* tetraploid varieties and to the development of an *in vitro* immature embryo rescue method (Arnau et al., 2006; Abraham and Arnau, 2009). Intercytotype crosses between diploid females and tetraploid males revealed the phenomenon of endosperm incompatibilitybecause they produce non-viable mature seeds with shrivelled endosperm. Obtaining tetraploid hybrids from intercytotype crosses suggested the ability of diploid progenitors to produce unreduced (2*n*) gametes, pollen or ovules. In the case of *D. alata*, triploid males are sterile because their flower buds remain unopened until drying off (Abraham and Nair, 1991) and the fertility of female flowers has never been demonstrated.

Flow cytometry is a fast and easy technique to determine ploidy levels in plants (Dolezela et al., 1994; Seker et al., 2003) and has already been used to screen 2*x* x 2*x* *D. alata* progenies (Arnau et al., 2011). Flow cytometry has also been used to determine endosperm ploidy in several species and to better understand the phenomenon of endosperm incompatibility in interspecific or intercytotype crosses (Pichot and Maataoui, 1997; Sliwinska et al., 2005).

Because microsatellite markers are co-dominant and highly reproducible, they are suitable for the analysis of allele segregation in progenies (Ashley and Dow, 1994). Once maternal and paternal microsatellite genotypes are known, the different pathways for gamete production and unions that give rise to polyploid individuals can be compared for their likelihood. For example, the origin of 2*n* gametes (maternal or paternal) can be deduced from microsatellite analysis. The transmitted heterozygosity can also provide knowledge about the type of mechanisms involved in the non-reduction, i.e., the suppression of the first or the second division restitution.

Implication of gametic non-reduction in the formation of polyploid individuals has never been demonstrated in *D. alata*. Furthermore, endosperm incompatibilities in this species have never been studied, in spite of their importance in intercytotype crosses. This knowledge is crucial for the optimisation of the production of new polyploid (3*x* and 4*x*) cultivars.

The objective of the present study was to use flow cytometry and microsatellite markers to understand the origin of *D. alata* spontaneous triploids and tetraploids. Diploid genitors suspected of producing unreduced gametes were used. Intercytotype crosses between diploid, triploids and tetraploid genitors generated different types of progenies. Flow cytometry was used to measure embryo and endosperm ploidy. Microsatellite markers were genotyped on

diploid parents and their detected polyploid offspring. This work made it possible to identify the origin of 2*n* gametes and endosperm, and provided knowledge about the formation of *D. alata* polyploids.

MATERIALS AND METHODS

Plant materials

A first progeny of 300 seeds was analysed. This progeny was obtained by crossing two diploid parents ($2n = 2x = 40$) (female "5F" and male "Kabusa"). The diploid status of 5F and Kabusa was ascertained by flow cytometry in Arnau et al. (2009). Both diploid genitors are suspected of producing unreduced gametes. Half of the seeds (N = 150) were sowed, and fresh leaves of seedlings were analysed using flow cytometry. The other half of the seeds was desiccated 90 days after pollination and the endosperm was separated from the embryo. Flow cytometry was then performed on embryos while a joint flow cytometry analysis was carried out on endosperm. SSR analyses were only performed on the leaves of detected triploid seedlings and embryos.

A second progeny of 2000 seeds obtained by crossing the diploid female clone 5F and the tetraploid male clone 148 was used: (i) to study endosperm incompatibility in $2x \times 4x$ crosses; and (ii) to analyse events of non-gametic reduction in the female parent. One thousand seeds were sowed to evaluate seed viability and to check the ploidy of emerging plantlets by flow cytometry. The remaining 1000 seeds were desiccated to allow a separate ploidy analysis on endosperm and embryos.

The gametic fertility of a triploid female clone (258F) was analysed by crossing it with the diploid male Kabusa. Obtaining tetraploids by this cross would be indicative of the formation of unreduced balanced gametes in triploid females. A total of 300 female flowers were fertilised by manual hybridisations, corresponding to 1800 potential seeds, given that a fruit can contain one to six seeds. The rates of fruit set and seed setting were recorded.

Flow cytometry analysis

Leaf flow cytometry analysis was performed as described by Arnau et al. (2009). The nuclear DNA content of samples was determined by comparison of the relative positions of the $G_{0\text{-}1}$ peak of different internal references: 760a as the triploid reference, 639a as the diploid reference and 754a as the tetraploid reference. The ploidy of these three clones was determined by mitotic chromosome counts in Arnau et al. (2009). For endosperm analysis, the protocol described by Sliwinska et al. (2005) was used with some adaptations. Endosperm was chopped up with a double-edged razor blade in 1 ml of nucleus isolation buffer (0.1 M

Tris-HCl, 2.5 mM $MgCl_2$. $6H_2O$, 85 mM NaCl, 1% w/v polyvinylpyrrolidone-PVP-10 and 0.1% v/v Triton X-100 pH 7). The suspension was filtered through a 30-µm-pore filter. Three hundred µl of filtrated endosperm solution, 200 µl of filtrated leaf solution of an internal standard and 400 µl of isolation buffer supplemented with propidium iodide (50 µg/ml) and ribonuclease A (50 µg/ml) were then mixed in a tube. The suspensions were incubated for approximately 5 min at room temperature. After incubation, each sample was run on a flow cytometer. DNA quantities were measured using a FACScalibur laser flow cytometer (Beckton Dickinson, USA) with Cellquest Software.

The choice of the internal reference was made according to the expected offspring or endosperm ploidy. To detect non-expected seedlings (triploid in a diploid population or tetraploid in a triploid population), an appropriate internal reference (diploid (639a) or triploid (760a), respectively) was used and results were interpreted as follows. The internal reference produces two fluorescence peaks: a major peak corresponding to 2*x* or 3*x* DNA quantities of the majority of leaf cells, and a replicated G2 minor peak corresponding to 4*x* or 6*x* DNA quantities from cells in mitotic interphase. When the fluorescence peak corresponding to the nuclei obtained from a given sampled individual was between these two reference peaks, the individual was assumed to be triploid with the diploid internal reference or tetraploid with the triploid internal reference if the sample peak is closer than the $G_{0\text{-}1}$ peak. When no additional peak was observed between the two peaks of the internal reference, the individual was assumed to have the same ploidy level of the reference. The same principle is applied to measured expected hexaploid (6*x*) endosperm. The tetraploid (754a) internal reference was used and produced two fluorescence peaks. When the fluorescence peak corresponding to the nuclei obtained from a given sampled endosperm was equidistant from these two reference peaks, the endosperm was assumed to be 6*x*.

Microsatellite amplification

Six SSR markers, Da2F10, Da1D08, mDaCIR8, mDaCIR60, mDaCIR61 and mDrCIR128, were selected from a set of 75 SSR developed from five different yam species: *D. rotundata*, *D. abyssinica*, *D. prahensilis*, *D. japonica* and *D. alata* (Misuki et al., 2005; Tostain et al., 2006; Andris et al., 2010). Primer sequences are given in Table 1. They are polymorphic between the diploid parents 5F and Kabusa, without any common allele. Da2F10, mDaCIR8, mDaCIR61 and mDrCIR128 show two alleles in the female progenitor 5F and two different alleles in the male progenitor Kabusa. Da1D08 and mDaCIR60 show heterozygosity in 5F and are homozygous in Kabusa but with different alleles.

Amplification was performed in a total volume of 20 µl containing 0.05 U/µl of polymerase Taq, 2 µl of 10X buffer, 0.2 mM of dNTP, 2 µmol of labelled or unlabelled primers, 2 mM of $MgCl_2$ and 10 ng of DNA. Forward primers were labelled with one of the following fluorophores: TET, NED, HEX or 6-FAM. PCR conditions were as follows: 5 min of denaturation at 94°C, followed by 30 cycles alternating 30 sec of denaturation at 94°C, 30 sec of hybridisation at annealing temperature, 35 sec of extension at 72°C, and ending with 5 min of final elongation at 72°C. PCR was carried out using a PTC100 thermocycler (MJ Research). Electrophoregrams were obtained by migration of amplification products on an ABI PRISM-TM 3100 automatic sequencer (Applied Biosystems). Allelic profiles were determined using GeneMapper v3.7 software (Applied Biosystems). Parents and polyploid zygotes eventually found in 150 seeds (second seed lot) of the 5F x Kabusa population were genotyped.

RESULTS

Triploids from the 2*x* x 2*x cross: 5F x Kabusa*

Frequency

Out of the 300 plantlets or embryos of the 5F x Kabusa progeny, four were found to be 3*x* (1.3%), and 296 were found to be 2*x* (98.7%). The diploid internal reference 639a was used for flow cytometry.

The aspect of all the sowed seeds that produce seedlings (diploid and triploid) was normal (plump) (Fig. 1a). No clear morphological differences made it possible to differentiate *a priori* 3*x* seeds from 2*x* seeds.

Endosperm ploidy and gamete origin

Out of the 150 seed lot for which endosperm was detached, flow cytometry was possible on two seeds that then produced 3*x* plants, hereafter referred to as 5K1 and 5K5. The diploid internal reference 639a was used for flow cytometry. Endosperm peaks for the two seeds corresponded to a 3*x* state (Fig. 2). Two other seeds that also produced 3*x* embryos could not be analysed because of a shrivelled endosperm (Fig. 1b). These two 3*x* individuals were obtained by embryo rescue and are hereafter referred to as 5K11 and 5K34.

Parental (5F and Kabusa), 5K1, 5K5, 5K11 and 5K34 genotypes for the six microsatellite markers are given in Table 2. Microsatellite analysis conclusions on the origin of the 3*x* individuals were drawn as follows. When the 3*x* offspring carried the two alleles of the female (5F) and only one allele from the male (Kabusa), it was assumed that the female produced an

unreduced ovule and the male a normal reduced pollen grain. When the two paternal alleles and only one maternal allele were transmitted, the unreduced gamete was assumed to be a pollen grain. On Fig. 3, the 5K34 seedling case is illustrated for locus mDaCIR8. The two male alleles (166 and 184 bp) are present in 5K34 with only one of the maternal alleles (171 bp). It can be postulated at this locus that 5K34 resulted from an unreduced pollen grain and a normal ovule. For other loci (mDaCIR61 and mDrCIR128), heterozygosity was not transmitted by the male or by the female.

Identically, triploid individuals obtained from plump seeds, 5K5 and 5K1, received heterozygosity from 5F on loci Da2F10 and Da1D08. It can be hypothesized that they arise from a non-reduced ovule (Table 2). Triploids obtained from shrivelled seeds, 5K34 and 5K11, are assumed to come from non–reduced pollen grains.

They are not the same loci that transmit male or female heterozygosity. On average, the rate of transmitted heterozygosity is 50% for 2*n* ovules (the case of 5K1 and 5K5) and approximately 22% for 2*n* pollen (5K11 and 5K34).

Offspring of the female 2x x male 4x cross

Out of the 1000 sowed seeds obtained by crossing the diploid female (5F) and the tetraploid male (148), 18 seedlings germinated from seeds with a plump aspect. Flow cytometry showed that these seedlings were all tetraploids. The triploid internal reference was used.

Out of the 1000 dessicated seeds, 995 had a shrivelled aspect and five were plump. Flow cytometry was carried out on 100 of the 995 shrivelled detached endosperms but only six had a sufficient signal. These six endosperms had a signal peak between the two reference peaks of the triploid internal reference "760a" (3*x* and 6*x* for the G2 peak). They were therefore assumed to be 4*x*. The corresponding embryos of these 100 shrivelled seeds were positioned as triploids using flow cytometry.

Flow cytometry analysis showed that embryos of the five plump seeds were tetraploid. The endosperm peak of these five seeds was located at a 6*x* position (Fig. 4) when the tetraploid internal reference was used.

Fertility of triploid females

A very small number of seeds were obtained by crossing the triploid female 258F with the diploid male Kabusa (only 18 out of an optimal number of 1800 seeds). Half (9) of these seeds lacked an embryo. The other half contained embryos but not one evolved into a viable seedling, even if development was initiated in two of them.

DISCUSSION

This work is the first study reported in Dioscoreacea where seeds were desiccated to allow separate ploidy analysis on endosperm and embryos. However, it appeared that if endosperm analysis is efficient for plump seeds, it is not easy to apply to shrivelled seeds. Because polyploids were obtained at a low rate, this could lead to doubts about the sample size of the progenies used to obtain accurate estimates of non-reduced gamete formation. Our results should be considered more as qualitative than accurate estimates.

In this study, 2*x* x 2*x* crosses gave 1.3% of 3*x* individuals with a contrasted endosperm aspect. The flow cytometry of joint embryos and endosperms, seed aspects and microsatellite analysis together led to evidence that both male and female clones produced 2*n* gametes that mated with *x* gametes to produce viable 3*x* plants. When the unreduced gamete was an ovule, endosperm ploidy was 3*x* and the seed was plump. The expected ploidy of endosperm in this case was 5*x* (4:1) and not the observed 3*x*. When the unreduced gamete was a pollen grain, endosperm was shrivelled and its ploidy level could not be determined.

In the same manner, 4*x* individuals were obtained in the 2*x* x 4*x* cross (1.15%). These seeds are easily identified by the plump aspect of their seeds, corresponding to the expected 6*x* endosperm if the diploid female produced a 2*n* ovule and if the endosperm originated from the fusion of the two 2*n* cells of the embryo sac (2*x*+2*x*) with the normal 2*x* spermatozoid produced by the 4*x* male. For the other seeds, embryos were 3*x* and the endosperm was shrivelled, consistent with the union of a normal reduced ovule (*x*) from the diploid female and a reduced pollen grain from the 4*x* male.

This suggests that the production of 2*n* gametes is at the origin of *D. alata* polyploids as is the case for potatoes (Iwanaga and Peloquin, 1982; Carputo et al., 2000) and many other species (see Ramsey and Schemske (1998) for a review).

Other phenomena such as polyspermy (fertilisation of an egg by two sperm nuclei) and endospermal polyembryony (formation of embryos from endosperm cells) have been suggested as possible mechanisms that could lead to the production of triploids in other species. Polyspermy is not considered as a likely mechanism in the formation of polyploids (Harlan and DeWet, 1998) but has been demonstrated in the genus *Juglans* (Navashin and Finn, 1951). In *D. alata*, neither polyspermy nor polyembryony can explain the microsatellite profiles obtained on the triploids for the six loci tested. Under the polyspermy hypothesis, regardless of the paternal genotype, individuals would carry only one of the paternal alleles at all loci because the two sperm nuclei are identical by mitosis (Fig. 5). For some loci, paternal

heterozygosity in triploids was observed and makes it possible to eliminate polyspermy. Endospermal polyembryony, which consists of the formation of embryos from endosperm cells, was observed in the genera Bracharia (Muniyamma, 1977), Beta (Yarmolyuk et al., 1990) and citrus (Gmitter et al., 1990). These examples are rare and weakly substantiated (Batygina and Vinogradova, 2007). In this case, regardless of the maternal genotype, individuals would carry only one maternal allele at all loci since the two polar nuclei are identical by mitosis. Because maternal heterozygosity was observed in our *D. alata* triploid individuals at some loci, polyembryony may also be eliminated.

All these elements make it possible to conclude that triploid *D. alata* originate from 2*n* gamete formation. Two basic types of meiotic restitution mechanisms that lead to 2*n* gamete formation have been reported: FDR and SDR (Mok and Peloquin, 1975; Park et al., 2007). These two cytological events do not transmit the same parental heterozygosity to their progeny. FDR mechanisms lead to 2*n* gametes that contain non-sister chromatids between the centromere and the first crossover. Consequently, all loci between the centromere and the first crossover that were heterozygous in the diploid parent will be heterozygous in the 2*n* gametes. Half of those beyond the crossover will be heterozygous in the gametes. SDR mechanisms lead to 2*n* gametes that contain sister chromatids between the centromere and the first crossover. All loci between the centromere and the first crossover that were heterozygous in the diploid parent will be homozygous in these 2*n* gametes, whereas those beyond the crossover will be heterozygous (Peloquin et al., 2008). As a result, heterozygosity transmission by FDR varies from 100 to 50%, and transmission by SDR varies from 0 to 100% and depends on the position of markers in relation to the centromere (Park et al., 2007).

Unreduced gametes were observed in the two sexes with a similar low probability (<2%). Microsatellite markers were used to detect non-gametic reduction in males and females by studying heterozygosity transmission. Our preliminary results would indicate that unreduced ovules could transmit more heterozygosity than unreduced pollen. However, our study clearly lacks the statistical power to draw a final conclusion: a greater number of 3*x* individuals should be genotyped on a greater number of SSR. Further analysis of PMCs (Pollen Mother Cells) could also help to determine the type of 2*n* gametes in males.

Unreduced gamete formation makes it possible to overcome post-zygotic barriers that occurred in interploid crosses due to endosperm abortion (Peloquin et al., 1999). Most of the expected endosperm ploidy levels were observed in the different crosses (♀2*x* (*n*) x ♂4*x*, (♀2*x* (2*n*) x ♂4*x*) with the remarkable exception of the 2*x* x 2*x* cross with the formation of 2*n*

ovules. In this latter case, the 3*x* individual resulted from plump seeds with a 3*x* endosperm. Histochemical analysis in *Dioscorea nipponica* by Torshilova et al. (2003) suggests that the embryo sac of Dioscoreacea would be of the monosporic octonuclear type, Polygonum, which leads to a 3*x* endosperm with a 2:1 ratio in a usual diploid cross. Therefore, the embryo sac of a diploid female that produces a 2*n* ovule is expected to contain two 2*n* polar nuclei. After fertilisation by a normal spermatozoid, these two polar nuclei should normally generate a non-viable 5*x* endosperm with a 4:1 ratio. Since the observed endosperm ploidy was 3*x*, this suggests that only one of the two polar nuclei could have been fertilised. This phenomenon has been observed in other species such as in *Triticum aestivum* (You and Jensen, 1985). For triploid individuals resulting from a union between unreduced pollen and a reduced ovule, endosperms were shrivelled. Their expected endosperm ratio is 2:2, which, unfortunately, could not be measured in our experiment. This suggests that this type of gametic combination should not lead to the production of viable seeds as a consequence of endosperm dysfunction.

No triploid seedlings were obtained from the 1000 seeds from the intercytotype cross, 2*x* female x 4*x* male. Embryos were 3*x* but their endosperm were found to be 4*x*, consistent with the expected 2:2 maternal to paternal genome ratio, confirming that in *D. alata,* unbalanced endosperm leads to shrivelled seeds and unviable seeds. Since *in vitro* embryo rescue on such crosses gives rise to 3*x* hybrids, this scenario is confirmed.

In brief, since plump seeds from 2*n* ovules x *n* pollen have a better chance of germinating in the wild than shrivelled seeds from an *n* ovule x 2*n* pollen, the likely spontaneous triploid formation in *D. alata* is the cross of a diploid maternal gamete with a haploid paternal gamete.

Since 3*x* are highly infertile, the existence of fertile 4*x* plants remains to be documented. Theoretically, the two different ways that could have led to the production of the first tetraploids are unilateral sexual polyploidisation (triploid bridge) and bilateral sexual polyploidisation (fusion of two unreduced gametes). Obtaining tetraploids via BSP has been demonstrated in several autopolyploid species such as red clover (Parrott and al., 1985) and *Dactylis glomerata* (Bretagnolle and Lumaret, 1995). In the 2*x* x 2*x* progeny, no tetraploid was obtained by bilateral sexual polyploidisation on the 300 seeds examined. With an observed rate of 2*n* gamete formation of 1.3%, the theoretical chance of obtaining a tetraploid would have been 1/2500. If this scenario could be verified by examining a much larger descent than in this study, it could be considered as much more likely than the triploid bridge

for *D. alata.* Furthermore, it would normally not have a problem of seed development with the BSP scenario because a normal 6*x* endosperm with a 2:1 (4:2) ratio is expected.

No tetraploid seedlings were obtained by triploid bridges on a descent of 2000 seeds (i.e., a cross between triploids and diploids to obtain tetraploids). Our results confirm a general picture of a very high sterility rate of triploid females, as reported by Abraham and Nair (1991). These authors concluded that triploid females are sterile based on a study carried out on 27 triploid females crossed with a male diploid (50-130 pollinations).

In triploids, segregation of chromosomes at meiosis is complex because of the trivalent formation at metaphase I, which would lead to a majority of aneuploid gametes after the second meiotic division. The probability of obtaining viable euploid gametes (balanced) depends on the *x* chromosome number. Given that each chromosome has a 50% chance of migrating to one of the cell's poles at Anaphase I, the frequency of obtaining haploids, diploids and triploids is $1/2^{x,}$ $1/2^{x}$ $+1/2^{x}$ and $1/2^{X}$ X3, respectively. The chromosome base number of most species for which the triploid bridge has been demonstrated is quite low. *Cucumis sativus,* in which two tetraploids were obtained by USP on 545 seeds has *x*=7 pairs of chromosomes (Diao et al., 2009).

It has been demonstrated that non-reduction gametic phenomena can increase the frequency of obtaining diploid and triploid gametes in triploids by SDR and FDR, respectively (Husband, 2004). This is the case for *C. angustifolium* (*x*=18) where the high rate of unreduced gametes made it possible to obtain tetraploids. The rate of 2*n* gametes observed in *C. angustifolium* species is ten times higher than that reported by Ramsey and Schemske (1998), based largely on crop plants. Moreover, in this species that has an Oenothera-type monosporic embryo sac, no endosperm incompatibility phenomenon was observed in intercytotype crosses (Husband, 2004). In *D. alata*, whose chromosome base number is *x*=20 (Arnau et al., 2009), viable haploid, diploid and triploid gametes are predicted at frequencies of $1/2^{20}$, $1/2^{20}$ + $1/2^{20}$ and $1/2^{20}$ x 3, respectively, corresponding to very low rates for obtaining viable seedlings. Screening of triploid females in other collections would make it possible to check if USP (triploid bridge) is a possible formation mechanism of tetraploids. However, in the CIRAD *D. alata* germplasm collection, no gametic non-reduction has yet been observed for triploid females (unpublished data) via USP checking. In *D. alata*, in addition to a very low theoretical frequency for obtaining triploid balanced gametes (three out of one million), it would be assumed that endosperm incompatibility would lead to a heptaploid endosperm with

a ratio of 6:1 (or tetraploid with a 3:1 ratio if only one polar nucleus is fertilised) and a likely non-viability for the seeds.

Moreover, our results revealed that in *D. alata*, like in other species (Hardy et al., 2001; Burton and Husband, 2001), tetraploids can be obtained by intercytotype crosses (2*x* x 4*x*) via gametic non-reduction in the diploid female progenitor. Thus, the gene flows between the diploid and tetraploid compartments may have contributed to enhancing diversity at the tetraploid level. Reciprocal crosses (4*x* x 2*x*), although not carried out in this study, could produce tetraploids via gametic non-reduction in the male diploid progenitor. Once the tetraploid pool is established, mating could start between 4*x* individuals and lead to the recombination of diversity at this ploidy level.

Conclusions

The major conclusion of this study is that polyploids in *D. alata* might have appeared as a result of 2*n* gamete formation. The most likely origin of spontaneous triploids would be the union of an unreduced egg and a reduced pollen grain with normal 3*x* endosperm, whose formation is still unknown. Crossing a diploid female with a tetraploid male is a possible way to obtain triploids in *D. alata* but requires the use of embryo rescue, excluding the possibility that this mechanism could have contributed to enhancing the diversity of the wild triploid pool. Although tetraploids were not obtained in this experiment, probably since the sample of seeds germinated was too small, we could reasonably assume that their most likely primary origin in the wild would be bilateral sexual polyploidisation via the mating of two unreduced gametes produced by diploids. Gene flows between diploid and tetraploid compartments by intercytotype crosses (2*x* x 4*x*) may have further contributed to broadening the allelic diversity at the tetraploid level, while recombination at the 4*x* level created new gene combinations. Screening of *D. alata* collections worldwide could make it possible to identify diploid progenitors with high 2*n* gamete production and to use them to enlarge the genetic diversity in the cultivated polyploid compartment. Knowledge of the origin of 2*n* gametes, SDR or FDR will make it possible to increase the transmission pathway of the parental heterozygosity rate.

LITERATURE CITED

Abraham K, Nair PG. 1991. Polyploidy and sterility in relation to sex in Dioscorea alata L. (Dioscoreaceae). *Genetica* **83**:93-97

Abraham K, Arnau G. 2009. Use of DNA markers for genetically improving the productivity, palatability, storability and dry matter content of tubers of greater yam. Report of project no. 3000-B1. Indo- French Society for the Promotion of Advanced Research (IFCPAR).New Delhi, India. 82 pp

Adams KL, Wendel JF. 2005. Polyploidy and genome evolution in plants. *Current Opinion in Plant Biology* **8**: 135-141

Andris M, Aradottir GI, Arnau G et al. 2010. Permanent Genetic Resources added to Molecular Ecology Resources Database 1 June 2010 – 31 July 2010. *Molecular Ecology Resources* **10**:1106-1108

Arnau G, Maledon E, Bachand I, Abraham K. 2006. Production of interploid hybrids and molecular markers heterozygosity determination using microsatellite markers in the greater yam, *D. alata*: Importance for the genetic improvement of the greater yam. In: Proceedings of 14th Triennial Symposium of the International Society for Tropical Roots Crops. 20-26 November 2006 Thiruvananthapuram, Kerala, India, 6 pp

Arnau G, Nemorin A, Maledon E, Abraham K. 2009. Revision of ploidy status of Dioscorea alata (Dioscoreaceae) by cytogenetic and microsatellite segregation analysis. *Theoretical and Applied Genetics* **118**: 1239-1249

Arnau G, Abraham K, Sheela MN, Chair H, Sartie A, Asiedu R. 2010. Yams. In: Bradshaw JE (ed) Root and tuber crops. Handbook of plant breeding Volume 7. Springer, pp 127-148

Arnau G, Nemorin A, Maledon E, Nudol E. 2011. Advances on polyploid breeding in yam D. alata. Procceding of the First International Symposium on Roots, Rhizomes, Tubers, Plantains, Bananas and Papaya . 7-10 November 2011 Santa Clara, Cuba, 4pp

Ashley MV, Dow BD.1994. The use of microsatellite analysis in population biology: background, methods and potential applications. EXS 69:185-201

Ayensu ES, Coursey DG. 1972. Guinea yams the botany, ethnobotany, use and possible future of yams in West Africa. *Economic botany* **26**: 301-318

Bakar SR. 1980. Chromosome behaviour in Nigerian yams (Dioscorea). *Genetica* **54**: 1-9

Batygina TB, Vinogradova Gyu. 2007. Phenomenon of Polyembryony. Genetic Heterogeneity of Seeds. *Russian Journal of Developmental Biology* **38**:126-151

Bousalem M, Arnau G, Hochu I, Arnolin R, Viader V, Santonini S, David J. 2006. Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam Dioscorea trifida and a new basic chromosome number in the Dioscoreae. *Theoretical and Applied Genetics* **113**:439-451

Bousalem M, Viader V, Mariac C, Gomez RM, Hochu I, Santoni S, David J. 2010. Evidence of diploidy in the wild Amerindian yam, a putative progenitor of the endangered species Dioscorea trifida (Dioscoreaceae). *Genome* **53**: 371-383

Burton TL, Husband BC. 2001. Fecundity and offspring ploidy in matings among diploid, triploid and tetraploid Chamerion angustifolium (Onagraceae): consequences for tetraploid establishment. *Heredity* **87**: 573–582

Bretagnolle F, Lumaret R. 1995. Bilateral polyploidization in Dactylis glomerata L. subsp.lusitanica: occurrence, morphological and genetic characteristics of first polyploids. *Euphytica* **84**: 197-207

Bretagnolle F, Thompson JD. 1995. Gametes with the somatic chromosome number: mechanisms of their formation and role in the evolution of autopolyploid plants. *New Phytology* **129**: 1-22

Carputo D, Barone A, Frusciante L. 2000. 2n gametes in the potato: essential ingredients for breeding and germplasm transfer. *Theoretical and Applied Genetics* **101**: 805-813

Comai L. 2005. The advantages and disadvantages of being polyploid. *Natural Revue of Genetics* **6**: 836-846

Costa LM, Gutièrrez-Marcos JF, Dickinson HG. 2004. More than a yolk: the short life and complex times of the plant endosperm. *Trends in Plant Science* **9**: 507-514

Den Nijs TPM, Peloquin SJ. 1977. Polyploid evolution via 2n gametes. *American Journal of Potato Research* **54**: 377-386

Diao WP, Bao SY, Jiang B, Cui L, Chen JF. 2009. Primary trisomics obtained from autotriploid by diploid reciprocal crosses in cucumber. *Sexual Plant Reproduction* **22**: 45-51

Doležela J, Lucrettib S, Schubertc I. 1994. Plant Chromosome Analysis and Sorting by Flow Cytometry. *Critical Reviews in Plant Sciences* **13**: 275-309

Egesi CN, Asiedu R. 2002. Analysis of yam yields using the Additive Main Effects and Multiplicative Interaction (AMMI) model. *African Crop Science Journal* **10**: 195-201

Gallais A. 2003. Quantitative genetics and breeding methods in autopolyploid plants. Editions Quae, 516 p.

Gmitter FG, Ling XB , Deng XX. 1990. Induction of triploid Citrus plants from endosperm calli in vitro. *Theoretical and Applied Genetics* **80**: 785-790

Hardy OJ , De Loose M , Vekemans X, Meerts P. 2001. Allozyme segregation and inter-cytotype reproductive barriers in the polyploid complex Centaurea jacea. *Heredity* **87**: 136–145

Harlan JR, deWet JMJ. 1975. On Ö. Winge and a Prayer: The origins of polyploidy. *The Botanical Review* **41**: 361-390

Husband BC. 2004. The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. *Biological Journal of Linnean Society* **82**: 537–546

Hutten RCB, Schippers MGM, Hermsen JGTh, Jacobsen E. 1995. Comparative performance of diploid and tetraploid progenies from 2x.2x crosses in potato. *Euphytica* **81**: 187-192

Iwanaga M, Peloquin SJ. 1982. Origin and evolution of cultivated tetraploid potatoes via 2n gametes. *Theoretical and Applied Genetics* **61**: 161-169

Navashin SG, Finn VV. 1951. On the history of the development of the chalazogamies Juglans nigra and J. regia. Izbrannye trudy, Moscow-Leningrad 1: 271-325

Köhler C, Scheid OM, Erilova A. 2010. The impact of the triploidblock on the origin and evolution of polyploid plants. *Trends in genetics* **26**: 142-148

Lange W, Wagenvoort M. 1973. Meiosis in triploid Solanum tuberosum L.*Euphytica* **22**: 8-18

Lebot V. 2009. Tropical root and tuber crops: cassava, sweet potato, yams and aroids. Wallingford (ed) CABI, XIX, 413 pp.

Malapa R, Arnau G, Noyer JL, Lebot V. 2005. Genetic diversity of the greater yam (Dioscorea alata L.) and relatedness to D. nummularia Lam. and D. transversa Br. as revealed with AFLP markers. *Genetic Resources Crop Evolution* **52**: 919–929

Mendiburu AO, Peloquin SJ. 1977. Bilateral sexual polyploidization in potatoes. *Euphytica* **26**: 573-583

Misuki I, Tani N, Ishida K, Tsumura Y. 2005. Development and characterization of microsatellite markers in a clonal plant, Dioscorea japonica Thunb. *Molecular Ecology Notes* **5**:721–723

Mizuochi H, Matsuzaki H, Moue T, Okazaki K. 2009. Diploid endosperm formation in Tulipa spp. and identification of a 1:1 maternal-to-paternal genome ratio in endosperms of T. gesneriana L. *Sexual plant reproduction* **22**: 27-36

Mok DWS, Peloquin SJ. 1975. Three mechanisms of 2n pollen formation in diploid potatoes. *Journal canadien de génétique et de cytologie* **17** : 217-225

Muniyamma M. 1977. Triploid embryo from endosperm in vivo. *Annals of Botany* **41**: 1077-1079

Nemorin A, Abraham K, David J, Arnau G. 2012. Inheritance pattern of tetraploid Dioscorea alata and evidence of double reduction using microsatellite marker segregation analysis. *Molecular Breeding* DOI: 10.1007/s11032-012-9749-0

Ortiz R, Vuylsteke D. 1995. Factors Influencing Seed Set in Triploid Musa spp. L. and Production of Euploid Hybrids. *Annals of Botany* **75** : 151-155

Parisod C, Holderegger R, Brochmann C. 2010. Evolutionary consequences of autopolyploidy. *New Phytology* **186**: 5–17

Park TH, Kim JB, Hutten RCB, van Eck HJ, Jacobsen E,Visser RGF. 2007. Genetic Positioning of Centromeres Using Half-Tetrad Analysis in a 4x–2x Cross Population of Potato. *Genetics* **176** : 85-94

Park SM, Wakana A, Kim JH, Jeong CS. 2002. Male and female fertility in triploid grapes (Vitis complex) with special reference to the production of aneuploid plants. *Vitis* **41**: 11-19

Parrott WA, Smith RR, Smith MM. 1985. Bilateral sexual tetraploidization in red clover. *Canadian Journal of Genetics and Cytology* **27**: 64-68

Paterson AH. 2005. Polyploidy, evolutionary opportunity, and crop adaptation. *Genetica* **123**: 191–196

Peloquin SJ, Boiteux LS, Carputo D. 1999. Meiotic Mutants in Potato: Valuable Variants. *Genetics* **153**: 1493-1499

Peloquin SJ, Boiteux LS, Simon PW, Jansky SH. 2008. A Chromosome-Specific Estimate of Transmission of Heterozygosity by 2n Gametes in Potato. *Journal of Heredity* **99**: 177-181

Pichot C, El Maataoui M. 1997. Flow cytometric evidence for multiple ploidy levels in the endosperm of some gymnosperm species. *Theoretical and Applied Genetics* **94**: 865-870

Ramachandran K.1968. Cytological studies in Dioscoreaceae. *Cytologia* **33**: 401-410

Ramanna MS, Jacobsen E. 2003. Relevance of sexual polyploidization for crop improvement – A review. *Euphytica* **133**: 3-8

Ramsey J, Schemske DW. 1998. Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants. *Annual Review of Ecology and Systematics* **29**: 467-501

Ramsey J. 2007. Unreduced gametes and neopolyploids in natural populations of Achillea borealis (Asteraceae). *Heredity* **98**: 143–150

Rieseberg LH, Willis JH. 2007. Plant speciation. *Science* **317**: 910-914

Scott RJ, Spielman M, Bailey J, Dickinson HG. 1998. Parent-of-origin effects on seed development in Arabidopsis thaliana. *Development* **125**: 3329-3341

Seker M, Tuzcu O, Ollitrault P. 2003. Comparison of nuclear DNA content of citrus rootstock populations by flow cytometry analysis. *Plant Breeding* **122**: 169–172

Sliwinska E, Zielinska E, Jedrzejczyk I. 2005. Are seeds suitable for flow cytometric estimation of plant genome size?. *Cytometry* **64**: 72-79

Soltis DE, Soltis PS, Tate JA. 2004. Advances in the study of polyploidy since Plant speciation. *New Phytologist* **161**: 173–191

Stupar RM, Bhaskar PB, Yandell BScet al. 2007. Phenotypic and Transcriptomic Changes Associated With Potato Autopolyploidization. *Genetics***176**:2055-2067

Torshilova AA, Titova GE, Batygina TB. 2003. Female reproductive structures and seed development in Dioscorea Nipponicamakino (Dioscoreaceae). *Acta Biologica Cracoviensia Series Botanica* **45**: 149–154

Tostain S, Scarcelli N, Brottier P, Marchand JL, Pham JL, Noyer JL. 2006. Development of DNA microsatellite markers in tropical yam (Dioscorea sp.). *Molecular Ecology Notes* **6**:173–175

Williams JH, Friedman WE. 2002. Identification of diploid endosperm in an early angiosperm lineage. *Nature* **415**: 522-526

Woodhouse M, Burkart-Waco D, Comai L. 2009. Polyploidy. Nature Education 2

Yarmolyuk GI, Shiryaeva GI, Kulik AG. 1990. Endosperm embryony- a new form of apomixes in Sugar Beet. Dokl. *VASKhNIL* **3**: 8-11

You R, Jensen WA. 1985. Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (Triticum aestivum). *Canadian Journal of Botany* **63**: 163-178

Table 1 Primer sequence of the six microsatellite markers using for study of allelic contribution of progenitor. Forward sequence (sequence F) and Reverse sequence (sequence R) are given.

Locus	Sequence F	Sequence R
Da2F10	TCAAGGATAAGAACTCCC	CAACGGCTAAACAGAAA
Da1D08	GATGCTATGAACACAACTAAC	TTTGACAGTGAGAATGGA
mDaCIR8	ACAGCAGCAAAATAACTG	TCTTTGCAGGAGAAGAGG
mDaCIR60	CAAAGACCAGGGAATGTG	AGAATGCAGAGCATGGTG
mDaCIR61	CTAACCCCTCCAAAGCTG	GGGCATTCACGTCTTTAT
mDrCIR128	CCGTATTCCAAGCGATAA	AGCGTGAAAACCTGATAAAA

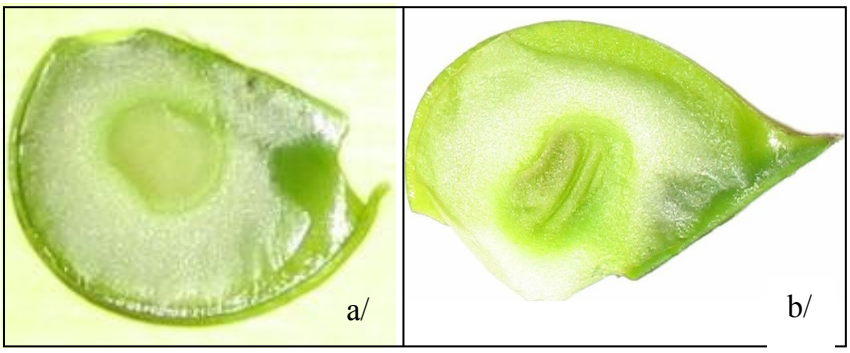


Figure 1 Photographs of a plump seed in a/ and shriveled seed in b/.

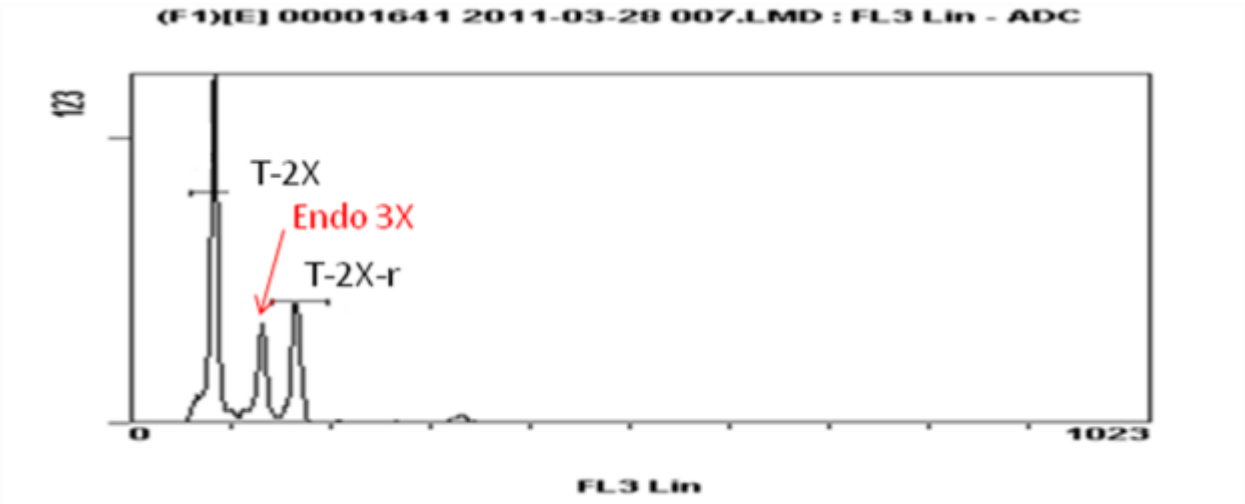


Figure 2 Flow cytometry analysis of detached endosperm from desiccated seeds from which grew triploid plants. Seeds were obtained on the 5F x Kabusa cross, both 2X parents.

The diploid internal reference (639a) produced two fluorescence peaks: (T-2X) the major G_{0-1} peak corresponding to a 2X DNA quantity of the majority of leave cells and (T-2X-r) the replicated G2 minor peak corresponding to the 4 X DNA quantities of cells in mitotic interphase. The fluorescent peak corresponding to endosperm (Endo 3X) is equidistant to the two reference peaks and is triploid (3n).

Table 2. Parental and hybrid genotypes at six SSR markers. The female progenitor "5F", the male progenitor "Kabusa" and four of their offspring 5K1, 5K5, 5K11 and 5K34 were analysed (out of a 300 progeny) . Alleles are given in bp.

	Da2F10	Da1D08	mDaCIR8	mDaCIR60	mDaCIR61	mDrCIR128
5F	126/132	303/313	171/188	146/157	200/217	286/310
Kabusa	124/130	306	166/184	142	188/193	300/308
5K5	_	_	166/171	142/146/157	188/200/217	308/310
5K34	_	303/306	166/171/184	142/146	188/200	300/310
5K1	124/126/132	303/306/313	166/171	_	_	300/310
5K11	124/132	_	166/184/188	142/146	_	286/308

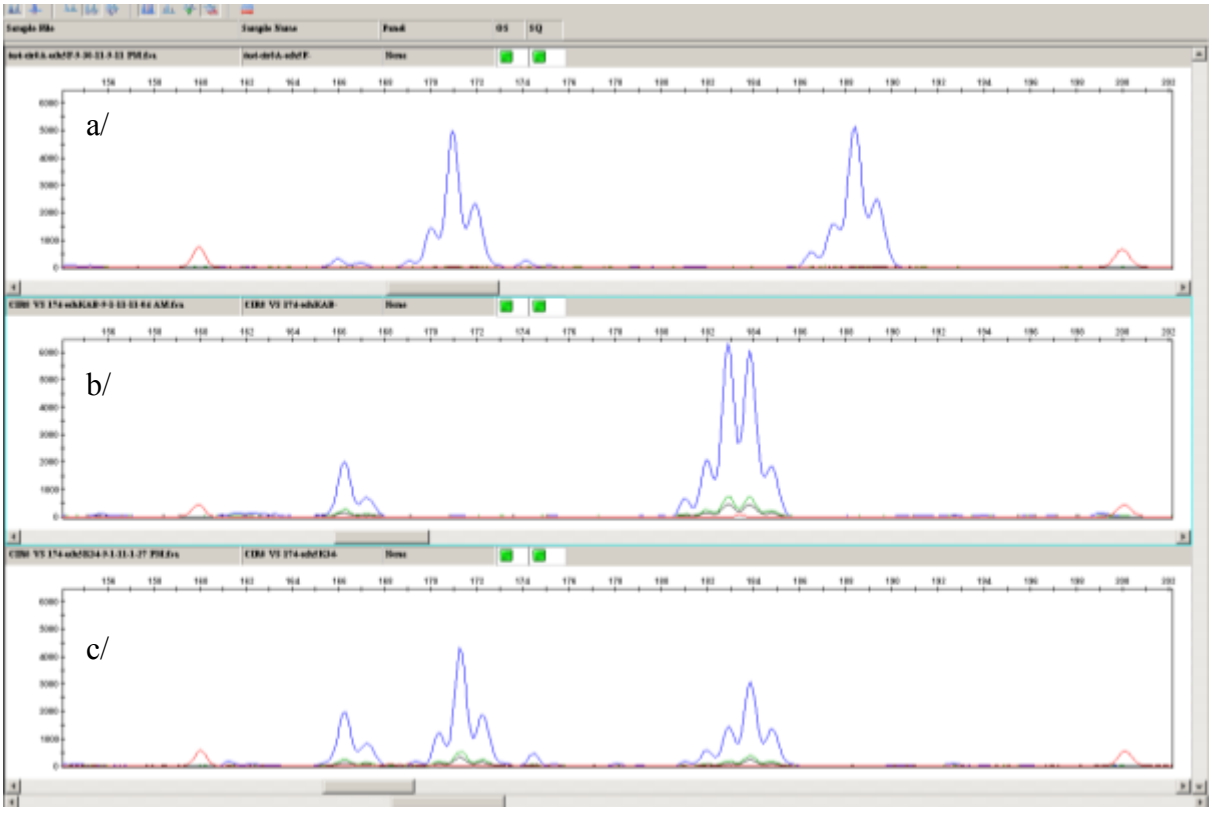


Figure 3 Phenomenon of non reduction of the male gamete

Electrophoregram at locus CIR8 a/for the diploid female progenitor 5F, b/ for the diploid male progenitor Kabusa and c/ for one triploid hybrid 5K34 with shrivelled endosperm

5F have phenotype 171/188; Kabusa have phenotype 166/184; 5K34 have phenotype **166**/171/**184**; paternal allelic contribution is in bold type.

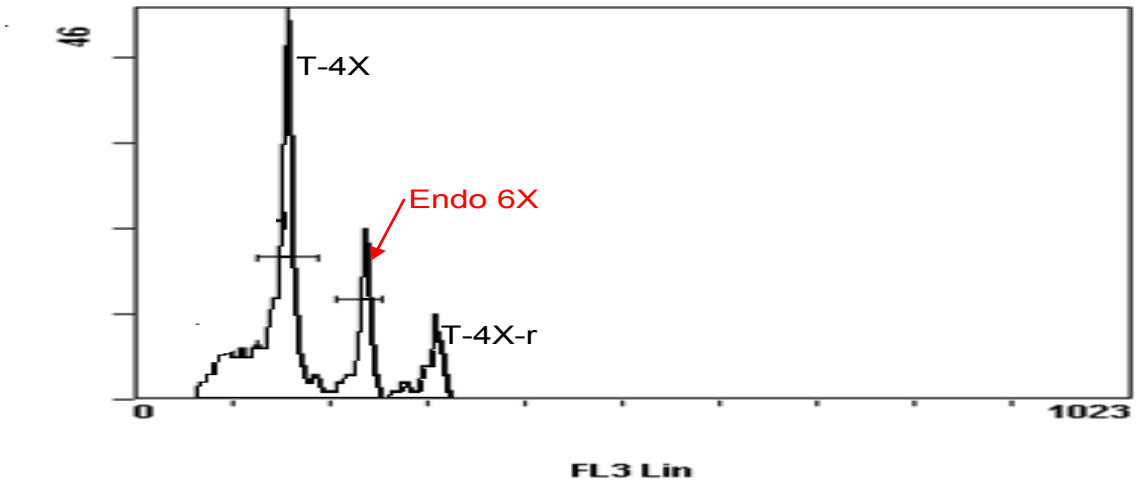


Figure 4 Flow cytometry analysis of detached endosperm from desiccated seeds from which grew tetraploid plants. Seeds were obtained on the 5F (2X) x 148 (4X) cross.

The tetraploid internal reference (754a) produced two fluorescence peaks: (T-4X) the major G_{0-1} peak corresponding to a 4X DNA quantity of the majority of leave cells and (T-4X-r) the replicated G2 minor peak corresponding to the 8 X DNA quantities of cells in mitotic interphase. The fluorescent peak corresponding to endosperm (Endo 6X) is equidistant to the two reference peaks and is hexaploid (6n).

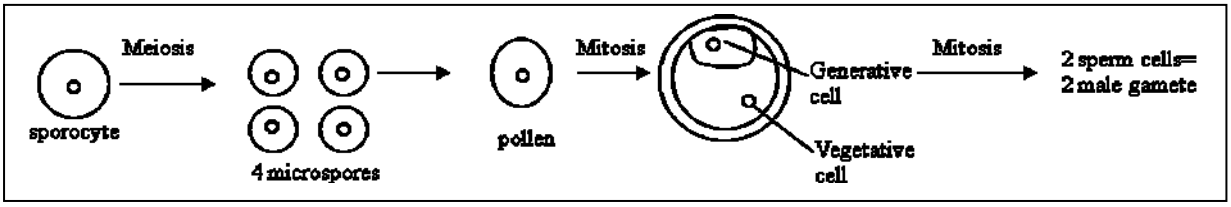


Figure 5 Microsporogenesis and microgametogenesis achieving formation of the two male gametes

DISCUSSION GENERALE-CONCLUSION

Au cours de la première partie de ce travail, différentes approches ont été utilisées pour déterminer le type de ségrégation des variétés tétraploïdes.

Des analyses d'hérédité de marqueurs microsatellites sur une descendance tétraploïde en utilisant la méthode Bayésienne, ont montré que les variétés tétraploïdes de *D. alata* ont clairement un type de ségrégation tétrasomique et sont par conséquent autotétraploïdes. En effet les facteurs de Bayes de l'ensemble des locus analysés étaient en faveur de l'autotétraploïdie avec un modèle mutationnel sans homoplasie. Nos résultats confirment que l'utilisation de la méthode bayésienne est un outil puissant pour déterminer le type d'hérédité chromosomique comme démontré chez d'autres espèces telle que l'espèce allotétraploïde *Borderea spp*. (Catalàn et al. 2006). Si l'allotétraploïdie segmentaire a été observée chez d'autres espèces telles que la canne à sucre (Janoo et al. 2004) et citrus (Kamiri et al. 2011), elle semble peu probable chez *D. alata*, car aucun des locus étudiés n'a donné de résultats significatifs en faveur de l'allopolyploïdie. Toutefois il est possible que certaines parties du génome puissent présenter des appariements homéologues. C'est pourquoi il serait intéressant d'augmenter le nombre de marqueurs afin de mieux couvrir l'ensemble du génome.

L'observation de double réduction sur 16 des 20 locus analysés confirme que le type de ségrégation est tétrasomique. En effet la double réduction est spécifique des autotétraploïdes vu qu'elle requière la formation de tétravalents (Ronfort et al. 1998 ; Luo et al. 2006).

L'étude des méioses des cellules mères de pollen (PMCs) de clones tétraploïdes a permis d'observer la formation d'un faible nombre de tétravalents (6-8).

En conclusion, l'ensemble des résultats obtenus (ségrégation polysomique, phénomènes de double réduction et formation de tétravalents) permet de conclure que les clones tétraploïdes sont autotétraploïdes. L'autotétraploïdie a été récemment démontrée chez une autre importante espèce cultivée du genre Dioscorea, *D. trifida* (Bousalem et al.2006).

Les connaissances acquises sur le type d'hérédité des variétés tétraploïdes ont des implications importantes en amélioration génétique. Etant donné que l'héritabilité des caractères dépend de la variance génétique et que des variances plus élevées sont attendues chez les autopolypoïdes par rapport aux allopolyploïdes, les caractères d'intérêt agronomique devraient être plus héritables. De plus, le progrès génétique devrait être supérieur en présence d'épistasie par rapport aux allopolyploïdes (Gallais 2003).

La seconde partie de la thèse a permis d'acquérir des connaissances importantes sur les mécanismes à l'origine des polyploïdes spontanés qui permettront d'optimiser les stratégies de production d'hybrides polyploïdes.

L'obtention de triploïdes au sein de descendances 2*x* x 2*x* et de tétraploïdes au sein de descendances 2*x* x 4*x*, décelés à l'aide de la cytométrie en flux (Arnau et al. 2006), avait permis de suggérer que la formation de gamètes non réduits (diplogamètes) soit à l'origine des phénomènes de polyploïdisation chez *D.alata*.

Ce travail a permis de prouver que des phénomènes de formation de diplogamètes ont eu lieu à la fois par voie mâle et femelle dans une descendance 2*x* x 2*x*, par des analyses de transmission de l'hétérozygotie parentale à l'aide de marqueurs microsatellites.

Par ailleurs, les analyses microsatellites ont permis également d'écarter la possible origine des triploïdes via des phénomènes de polyspermie (fertilisation d'une oosphère par deux noyaux mâles) et de polyembryonie (formation de l'embryon à partir de cellules de l'albumen).

Une des originalités de ce travail réside dans l'utilisation de la cytométrie en flux pour déterminer les niveaux de ploïdie de l'albumen et comprendre les phénomènes d'incompatibilités observés lors des différents croisements.

L'analyse conjointe de la ploïdie des albumens et de l'aspect des graines des triploïdes issus du croisement 2*x* x 2*x* permet de conclure que l'origine la plus probable des triploïdes naturels serait l'union d'un ovule non réduit avec un pollen réduit. Théoriquement, lors de la formation de 2n gamètes chez la femelle, l'albumen devrait être pentaploïde (ratio 4 :1). Les résultats obtenus montrent qu'il est triploïde (ratio 2 :1), suggérant une fécondation d'un seul noyau polaire, phénomène ayant déjà été observé chez *Triticum aestivum* (You and Jensen 1985). Des analyses microsatellites sur de tels albumens devraient permettre de confirmer cette hypothèse. Par ailleurs, l'aspect des graines était normal et de tels hybrides ont pu être obtenus par semis ce qui confirme l'existence d'un ratio équilibré 2/1 au sein de ces albumens.

Les résultats obtenus montrent qu'il est peu probable que le phénomène de diplogamétisation chez les mâles soit à l'origine des triploïdes naturels étant donné qu'il produit des graines non viables (fripées) avec un albumen théorique tétraploïde (ratio 2 :2).

Par ailleurs les croisements 2*x* x 4*x* ne peuvent pas être à l'origine des triploïdes spontanés du fait qu'ils produisent des graines fripées dont le niveau de ploïdie de l'albumen est tétraploïde avec un rapport déséquilibré 2/2. Des études antérieures ont démontrés que le croisement réciproque 4*x* x 2*x* ne produit pas de graines, ce qui s'expliquerait par le fait que l'albumen serait pentaploïde avec un rapport déséquilibré 4/1. Nos résultats montrent que ces combinaisons gamétiques n'ont pas pu contribuer à enrichir la diversité du pool triploïde naturel.

Théoriquement les deux voies de production des premiers tétraploïdes naturels sont la polyploidisation sexuelle bilatérale (BSP) et la polyploidisation sexuelle unilatérale (USP). La voie de production la plus probable chez *D. alata* serait la BSP comme chez le trèfle rouge (Parrot et al. 1985) et *Dactylis glomerata* (Bretagnole et Lumarett 1995). En effet bien qu'aucun hybride tétraploïde n'ait été obtenu à partir du croisement 2*x* x 2*x* dont les géniteurs étaient suspectés de produire des 2n gamètes, on aurait pu en obtenir à un taux de 1/2500 étant donné les pourcentages de formation de 2n gamètes observés dans notre étude. L'USP bien qu'observé chez d'autres espèces tel que *C. angustifolium* (Husband 2004) ou *Cucumis sativus* (Diao et al. 2009), reste fort peu probable dans le cas de *D. alata* du fait de la stérilité des mâles triploïdes dont les fleurs restent fermées jusqu'à dessèchement (Abraham et Nair 1991) et des femelles triploïdes montré dans notre étude, incapables de produire des graines viables par croisement avec des mâles diploïdes et tétraploïdes. La stérilité des femelles triploïdes s'expliquerait par leurs faibles taux de production de gamètes équilibrés (10^{-6} pour les gamètes 2*x* et 10^{-7} pour les gamètes 3*x*) du fait du nombre chromosomique de base *x*=20 élevé de *D.alata*. Chez certaines espèces la formation de gamètes non réduits par restitution de première division (FDR) augmente le taux de formation de gamètes équilibrés comme c'est le cas chez *C. angustifolium* (Husband 2004) où le bridge triploïde a été démontré. Cependant ce phénomène n'a pas été observé dans le cadre de notre étude.

L'origine la plus probable des variétés tétraploïdes serait donc la formation de gamètes non réduits (2n gamètes) chez des diploïdes, en une seule étape (Polyploidïsation sexuelle bilatérale).

La diversification du pool tétraploïde a pu être réalisé via des croisements interploïdes (2*x* x 4*x* et 4*x* x 2*x)* et par croisements entre tétraploïdes (4*x* x 4*x*). En effet le croisement 2*x* x 4*x*

produit des graines viables d'aspect normal, via la formation d'ovules non réduits chez la femelle diploïde, dont l'albumen est hexaploïde avec un ratio 2 :1 normal. Le croisement réciproque 4*x* x 2*x* avec formation de gamètes non réduits chez le mâle bien que non réalisé dans cette étude, devrait également donner des graines viables. Ainsi, des flux de gènes entre les compartiments diploïde et tétraploïde ont pu contribuer à augmenter la diversité au niveau tétraploïde.

En conclusion, les polyploïdes de *D.alata* seraient apparus par polyploïdisation sexuelle via la formation de gamètes non réduits. Le pool triploïde se serait édifié et diversifié uniquement par formation de gamètes non réduits chez les femelles diploïdes, du fait de la non viabilité des croisements avec formation de gamètes non réduits chez les mâles et des croisements interploïdes (2*x* x 4*x* et 4*x* x 2*x*). Le pool tétraploïde serait apparu par union de deux gamètes non réduits de géniteurs diploïdes, au sein d'une seule espèce du fait de l'autotétraploïde démontrée. Par la suite ce pool aurait été diversifié via des croisements interploïdes avec formation de 2n gamètes chez les femelles diploïdes et certainement chez les males, ainsi que par croisements au sein du pool 4X. Des évènements cytologiques de diploïdisation partielle des méioses au fil des générations auraient conduit à la production de gamètes génétiquement équilibrés expliquant la fertilité élevée des clones tétraploïdes.

Cette thèse a permis d'acquérir des connaissances sur les voies exploitables en amélioration pour la production de polyploïdes (Annexe 3). Trois voies peuvent être utilisées pour l'obtention de triploïdes : des croisements intraploïdes 2*x* × 2*x* avec formation de 2n ovule , des croisements intraploïdes 2*x* × 2*x* avec formation de diplogamètes chez le géniteur mâle et des croisements interploïdes 2*x* × 4*x*. Seule la première permet d'obtenir des graines viables, les deux autres requérant le sauvetage d'embryons immatures du fait d'incompatibilités au niveau de l'albumen. Des tétraploïdes peuvent être obtenus via trois voies différentes : des croisements interploïdes 2*x* × 4*x* avec formation de 2*n* ovule, des croisements interploïdes 4*x* × 2*x* avec formation de diplogmètes chez le géniteur mâle et des croisements intraploïdes 4*x* × 4*x*. Toutes ces voies produisent des graines viables du fait que la formation de 2n gamétophytes lors des croisements interploïdes, permet de surmonter les barrières post-zygotiques dues à des incompatibilités au niveau de l'albumen.

Etant donné que les croisements permettant la production de polyploïdes impliquent pour la plupart la formation de 2*n* gamètes, il serait intéressant d'explorer les collections mondiales à

la recherche de géniteurs à haut potentiel de formation de 2n gamètes à tous les niveaux de ploïdie.

L'acquisition de connaissances sur les mécanismes à l'origine de la formation des 2n gamètes (restitution de première division ou restitution de seconde division) est nécessaire afin de permettre de maximiser les taux d'hétérozygotie transmis aux hybrides polyploïdes.

Des prospections en vue de rechercher la forme diploïde sauvage des *D.alata* pourraient permettre d'exploiter les ressources génétiques du compartiment sauvage ainsi que d'étudier l'évolution du complexe dans les zones de sympatrie. Des études récentes chez l'espèce *D.trifida*, ont permis d'identifier la forme diploïde sauvage à l'origine de cette espèce tétraploïde, qui est en sympatrie avec des polyploïdes dans le centre d'origine (Bousalem et al.2010).

L'espèce polyploïde *D.nummularia* (3*x*, 4*x*, 5*x* et 6*x*), qui est celle qui est plus proche génétiquement de *D.alata* (Malapa et al. 2005) est intéressante à plusieurs niveaux. Elle présente une résistante totale à l'anthracnose, qui est la principale maladie chez *D.alata*, ainsi qu'une meilleure aptitude au désaisonnement (Malapa 2006). Des croisements interspécifiques avec cette espèce au niveau tétraploïde pourraient permettre d'introgresser ces caractères (Annexe 4).

Bibliographie

Abraham K, Nair SG, Sreekumari MT, Unnikrishnan M (1986) Seed set and seedling variation in greater yam (Dioscorea alata L.). Euphytica 35:337-343

Abraham K, Nair PG (1990) Floral biology and artificial pollination in Dioscorea alata L. Euphytica 48:45-51

Abraham K, Nair PG (1991) Polyploidy and sterility in relation to sex in Dioscorea alata L. (Dioscoreaceae). Genetica 83:93-97

Andris M, Aradottir GI, Arnau G et al (2010) Permanent Genetic Resources added to Molecular Ecology Resources Database 1 June 2010 – 31 July 2010. Mol Ecol Resour 10:1106-1108

Arnau G, Maledon E, Bachand I, Abraham K (2006). Production of interploid hybrids and molecular markers heterozygosity determination using microsatellite markers in the greater yam, *D. alata*: Importance for the genetic improvement of the greater yam. In: Proceedings of 14th Triennial Symposium of the International Society for Tropical Roots Crops. 20-26 November 2006 Thiruvananthapuram, Kerala, India, 6 pp

Arnau G, Nemorin A, Maledon E, Abraham K (2009) Revision of ploidy status of Dioscorea alata (Dioscoreaceae) by cytogenetic and microsatellite segregation analysis. Theor Appl Genet 118: 1239-1249

Arnau G, Abraham K, Sheela MN, Hana Chair, Alieu Sartie, Robert Asiedu (2010) Yams. In: Bradshaw JE (ed) Root and tuber crops. Handbook of plant breeding Volume 7. Springer, pp 127-148

Ayensu ES, Coursey DG (1972) Guinea yams the botany, ethnobotany, use and possible future of yams in West Africa. Economic botany 26: 301-318

Bousalem M, Arnau G, Hochu I, Arnolin R, Viader V, Santoni S, David J (2006) Microsatellite segregation analysis and cytogenetic evidence for tetrasomic inheritance in the American yam Dioscorea trifida and a new basic chromosome number in the Dioscoreae. Theor Appl Genet 113:439-451

Bretagnolle F, Lumaret R (1995) Bilateral polyploidization in Dactylis glomerata L. subsp.lusitanica: occurrence, morphological and genetic characteristics of first polyploids. Euphytica 84: 197-207

Burkill IH (1960) The organography and the evolution of *Dioscoreaceae*, the family of yam. J. Linn. Soc. (Bot.) 56 : 319-420

Carputo D, Barone A, Cardi T, Sebastiano A, Frusciante L, Peloquin SJ (1997) Endosperm balance number manipulation for direct in vivo germplasm introgression to potato from a

sexually isolated relative (Solanum commersonii Dun.). Proceedings of the National Academy of Sciences of the USA 94: 12013-12017

Catalán P, Segarra-Moragues JG, Palop-Esteban M, Moreno C, González-Candelas F (2006) A Bayesian Approach for Discriminating Among Alternative Inheritance Hypotheses in Plant Polyploids: The Allotetraploid Origin of Genus Borderea (Dioscoreaceae). Genetics172:1939-1953

Coursey DG (1967) Yams. Longmans, London 230pp.

Degras L (1993) The Yam, a Tropical Root Crop. MacMillan, London 408 pp

Diao WP, Bao SY, Jiang B, Cui L, Chen JF (2009) Primary trisomics obtained from autotriploid by diploid reciprocal crosses in cucumber. Sex Plant Reprod 22: 45-51

Essad S (1984)Geographical variation in base chromosome number and polyploidy within the genus Dioscorea, on the basis of chromosome count in Dioscorea transversa Brown, D. pilosiuscula Bert. and D. trifida L. Agronomie 4(7):611-617

Gallais A (2003) Quantitative genetics and breeding methods in autopolyploid plants. Editions Quae, 516 p.

Gamiette F, Bakry F, Ano G (1999) Ploidy determination of some yam species (Dioscorea spp.) by flow cytometry and conventional chromosome counting. Genet Resour Crop Evol 46:19-27

Hanneman RE, Peloquin SJ (1967) Crossability of 24-chromosome potato hybrids with 48-chromosome cultivars. European Potato Journal 10: 62-73

Haneman RE, Peloquin SJ (1968) Ploidy levels of progeny from diploid-tetraploid crosses in the potato. American Potato Journal 45: 255-261

Husband BC (2004) The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biol J Linnean Soc 82: 537–546

Jannoo N, Grivet L, David J, D'Hont A, Glaszmann JC (2004) Differential chromosome pairing affinities at meiosis in polyploid sugarcane revealed by molecular markers. Heredity 93: 460–467

Jansky SH, Yerk GL, Peloquin SJ (1990) The use of potato haploid to put 2x wild species germplasm in a usable form. Plant breeding 104: 290-294

Lebot V (2009) Tropical root and tuber crops: cassava, sweet potato, yams and aroids. Wallingford (ed) CABI, XIX, 413 pp.

Luo ZW et al. (2006) Constructing genetic linkage maps under a tetrasomic model. Genetics 172:2635–2645

Malapa R, Arnau G, Noyer JL, Lebot V (2005) Genetic diversity of the greater yam (Dioscorea alata L.) and relatedness to D. nummularia Lam. and D. transversa Br. as revealed with AFLP markers. Genet Resour Crop Evol 52:919–929

Marks GE (1966) The egnima of triploid potatoes. Euphytica 15: 285-290

Masterson J (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science 264: 421-424

Miège J (1952) Contribution à l'étude systématique des Dioscorea ouest africains. Th : Sciences Naturelles n° 6445. Université de Paris. 266pp.

Mignouna HD, Mank RA, Ellis THN, Van Den Bosch N, Asiedu R, Abang MM, Peleman J (2002) A genetic linkage map of water yam (Dioscorea alata L.) based on ALFP markers and QTL analysis for anthracnose resistance. Theor. Appl. Genet. 105 : 726-735

Mignouna HD et Dansi A (2003) Yam (Dioscorea ssp.) domestication by the Nago and Fon ethnic groups in Benin. Genetic Resources and Crop Evolution 50: 519-528

Misuki I, Tani N, Ishida K, Tsumura Y (2005) Development and characterization of microsatellite markers in a clonal plant, Dioscorea japonica Thunb. Mol Ecol Notes 5:721–723

Mok DWS, Peloquin SJ (1975) Three mechanisms of 2n pollen formation in diploid potatoes. Journal canadien de génétique et de cytologie 17 : 217-225

Parisod C, Holderegger R, Brochmann C (2010) Evolutionary consequences of autopolyploidy. New Phytol 186: 5–17

Park TH, Kim JB, Hutten RCB, van Eck HJ, Jacobsen E,Visser RGF (2007) Genetic Positioning of Centromeres Using Half-Tetrad Analysis in a 4x–2x Cross Population of Potato. Genetics 176 : 85-94

Parrott WA, Smith RR, Smith MM (1985) Bilateral sexual tetraploidization in red clover. Can. J. Genet. Cytol 27: 64-68

Peloquin SJ, Boiteux LS, Simon PW, Jansky SH (2008) A Chromosome-Specific Estimate of Transmission of Heterozygosity by 2n Gametes in Potato. J Hered 99: 177-181

Ramachandran K. (1968) Cytological studies in Dioscoreaceae. Cytologia 33: 401-410

Ramsey J, Schemske DW (1998) Pathways, Mechanisms, and Rates of Polyploid Formation in Flowering Plants. Annu Rev Ecol Syst 29: 467-501

Ramsey J (2007) Unreduced gametes and neopolyploids in natural populations of Achillea borealis (Asteraceae). Heredity 98: 143–150

Rao AN (1953) Embryology of Dioscorea, oppositifolia L. Phytomorph. 3. 121-126

Ronfort JL, Jenczewski E, Bataillon T, Rousset F (1998) Analysis of population structure in autotetraploid species. Genetics 150: 921–930

Scarcelli N, Daı¨nou O, Agbangla C, Tostain S, Pham JL (2005) Segregation patterns of isozyme loci and microsatellite markers show the diploidy of African yam Dioscorea rotundata (2n = 40). Theor Appl Genet 111:226–232

Segnou CA, Fatokun MO, Akoroda et S. K. Hahn (1992) Studies on the reproductive biology of white yam (Dioscorea rotundata Poir.) Euphytica 64: 197-203

Smith PM (1916) The Development of the Embryo and Seedling of Dioscorea villosa.

Bulletin of the Torrey Botanical Club 43: 545-558

Soltis DE, Soltis PS, Tate JA (2004) Advances in the study of polyploidy since Plant speciation. New Phytologist 161: 173–191

Soltis PS and. Soltis DE (2009) The Role of Hybridization in Plant Speciation. Annual Review of Plant Biology 60: 561-588

Stebbins GL (1950) Variation and evolution in plants. Columbia University Press, New York, 643 pp

Stelly DM, Peloquin SJ (1986) Formation of 2N megagametophytes in diploid tuber-bearing Solanums. American Journal of Botany 73: 1351-1363

Stift M, Berenos C, Kuperus P, van Tienderen PH (2008) Segregation models for disomic, tetrasomic and intermediate inheritance in tetraploids: a general procedure applied to Rorippa (yellow cress) microsatellite data. Genetics 179: 2113–2123

Torshilova AA, Titova GE, Batygina TB (2003) Female reproductive structures and seed development in Dioscorea Nipponicamakino (Dioscoreaceae). Acta Biologica Cracoviensia Series Botanica 45: 149–154

Tostain S, Scarcelli N, Brottier P, Marchand JL, Pham JL, Noyer JL (2006) Development of DNA microsatellite markers in tropical yam (Dioscorea sp.). Mol Ecol Notes 6:173–175

Ortiz R, Frusciante L, Carputo D (2005) Dedication: Stanley J. Peloquin potato geneticist and cytogeneticist. Plant Breeding Review DOI: 10.1002/9780470650301.ch1

Ortiz R, Simon P, Jansky S, Stelly D (2009) Ploidy manipulation of the gametophyte, endosperm and sporophyte in nature and for crop improvement: a tribute to Professor Stanley J. Peloquin (1921-2008). Annals of Botany 104: 795-807

Prain D, Burkill IH (1939) An account of the genus Dioscorea in the East. Part 2. The species which twine to the right: with addenda to Part 1 and a summary. Annals of the Royal Botanic Gardens, Calcutta, 14, part 2, 1939 pp.

Werner JE, Peloquin SJ (1991) Occurrence and mechanisms of 2n egg formation in 2x potato. Genome 34: 975-982

You R, Jensen WA (1985) Ultrastructural observations of the mature megagametophyte and the fertilization in wheat (Triticum aestivum). Canadian Journal of Botany 63 : 163-178

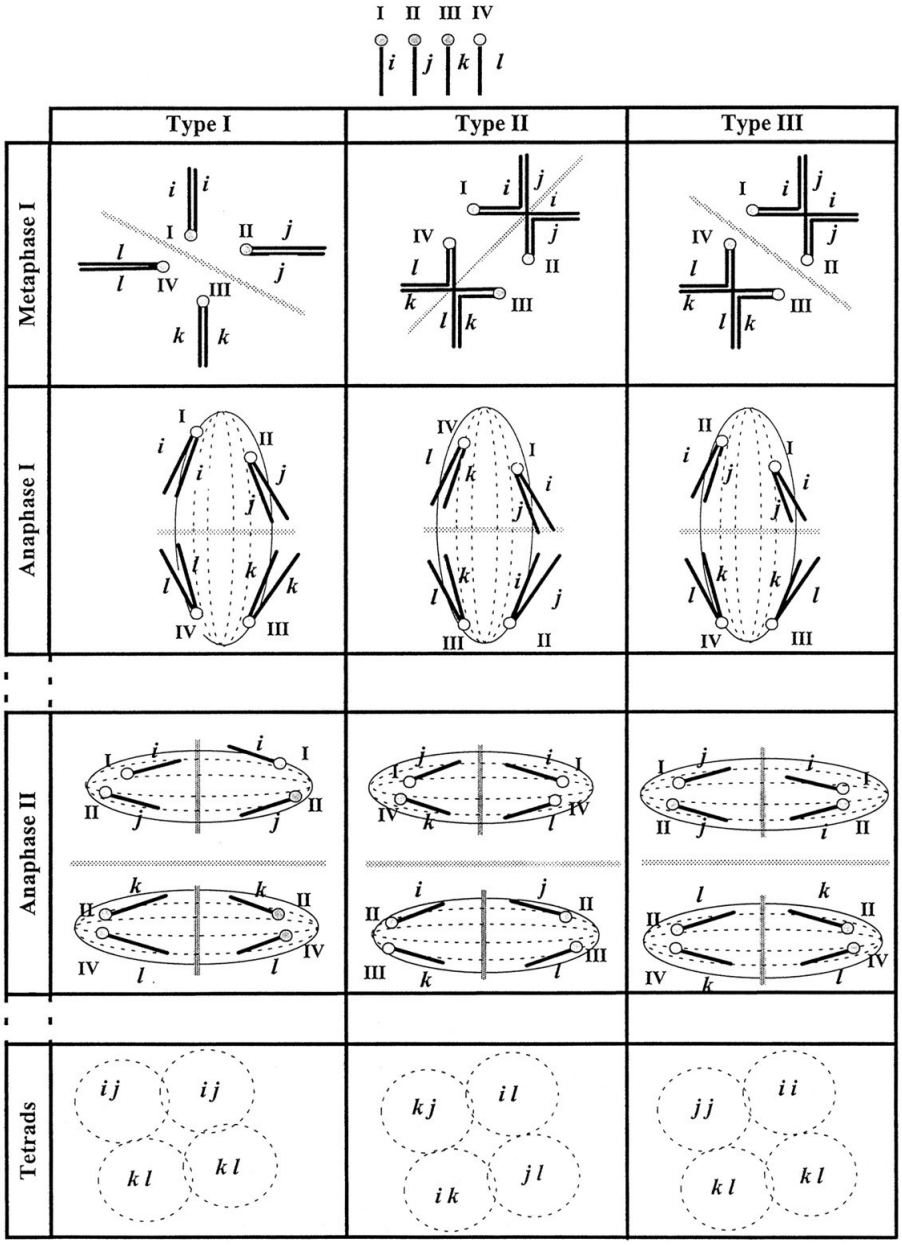
Annexes

Annexe 1 : Origine géographique des principales espèces cultivées de *Dioscorea* spp. (Arnau et al. 2010)

Table 4.1 Geographic origin of important cultivated *Dioscorea* spp.

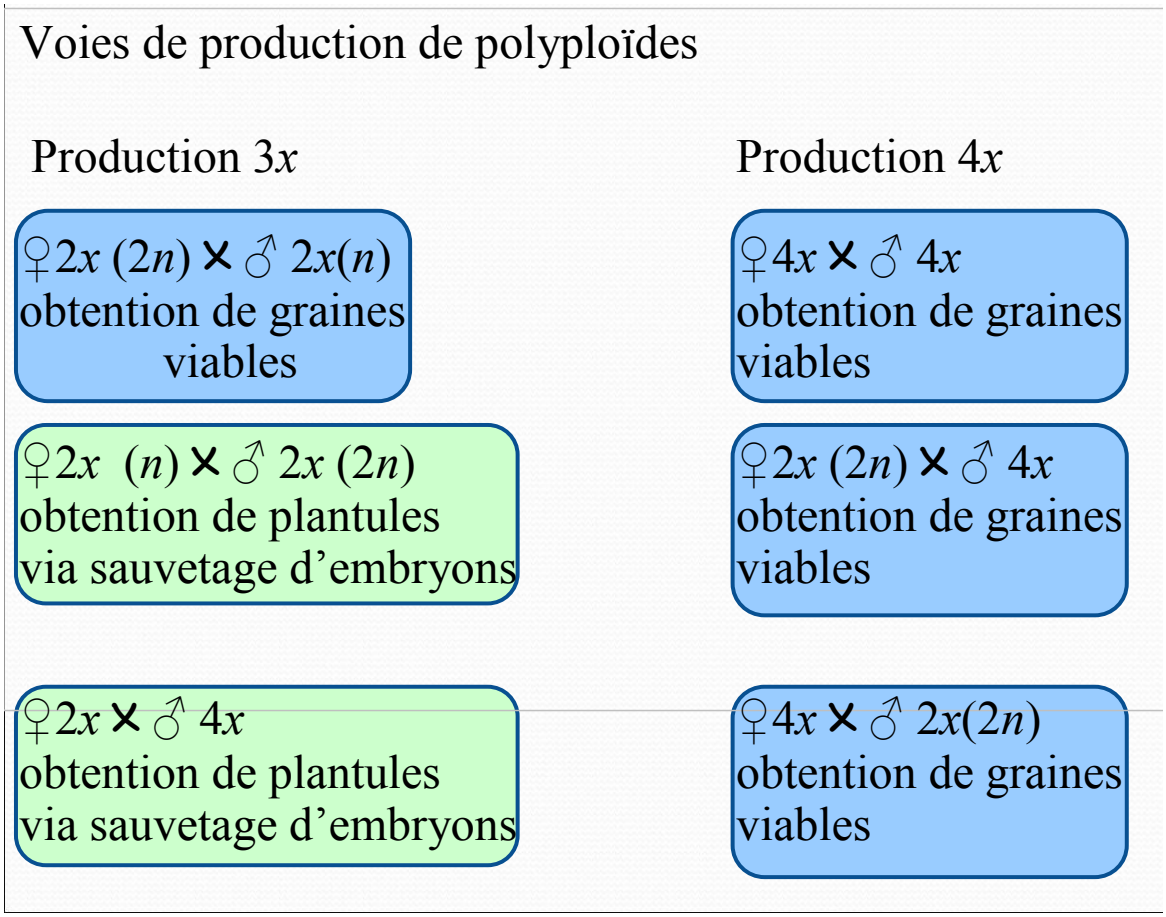
Dioscorea spp.	Common names	Botanical section	Geographic origin
Dioscorea alata	Greater, water, winged yam	Enantiophyllum	Southeast Asia, Melanesia
D. rotundata	White Guinea yam	Enantiophyllum	West Africa
D. cayenensis	Yellow Guinea yam	Enantiophyllum	West Africa
D. trifida	Aja, aje, cush–cush, yampi	Macrogynodium	South America
D. esculenta	Lesser yam, Asiatic yam	Combilium	Southeast Asia, Melanesia
D. oppositа-japonica	Chinese, Japanese yam	Enantiophyllum	Japan, China
D. bulbifera	Aerial, bulbil, bearing yam	Opsophyton	South America, Africa, Asia, Melanesia
D. nummularia	Spiny yam, wild yam	Enantiophyllum	Melanesia
D. transversa	Marou, wael	Enantiophyllum	Australia, Melanesia
D. pentaphylla	Five-leaved yam	Lasiophyton	Southeast Asia, Melanesia

Annexe 2 : Patrons d'hérédité possibles après formation de quadrivalent pour un individu autotétraploïde à un locus donné (Ronfort et al. 1998)



Possible segregation patterns of a locus in an autotetraploid individual following the formation of a quadrivalent. Type I describes the segregation patterns expected when there is no crossover between
the centromere and the locus. The first division is then reductional. When a crossover occurs between the centromere and the locus (Types II and III), the first division can be either equational (Type II) or reductional (Type III). Under Type III, the second division may then lead to double reduction. In the present case, gametes *ii* and *jj* have undergone double reduction.

Annexe 3 : Synthèse des voies de productions de polyploïdes



Annexe 4 Schéma d'amélioration élaboré à partir des connaissances acquises dans le cadre de cette thèse.